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Regulation of antioxidant defenses in the prevention of skeletal muscle deconditioning

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CERTIFICAN:

Que la presente memoria, titulada "**Regulation of antioxidant defenses in the prevention of skeletal muscle deconditioning**", corresponde al trabajo realizado bajo su dirección por D/Dña. **Coralie ARC-CHAGNAUD**, para su presentación como Tesis Doctoral en el Programa de Doctorado en Fisiología de la Universitat de València.

Y para que conste firma/n el presente certificado en Valencia, a 23 de septiembre de 2019.

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ABSTRACT

Musculoskeletal system plays a key role in organism's well-functioning and is responsible for a large variety of functions such as posture, locomotion, balance, and activities of daily life. The quality of the skeletal muscle is therefore capital to maintain quality of life and, in the long term, survival. Hypoactivity and aging are two situations that cause skeletal muscle deconditioning, therefore sharing common characteristics: loss of muscle strength, muscular atrophy and MyHC redistribution, as well as IMAT accumulation. To date, there is plenty of evidence supporting a causative link between oxidative stress phenomenon and muscle deconditioning.

The two studies exposed in this thesis demonstrated that modulate antioxidant defense systems have important consequences on deconditioned skeletal muscle tissue.

The first study aimed to evaluate frailty in old female animals, using WT and G6PD-overexpressing mice. We did a longitudinal functional evaluation, testing the mice each 2 months from 18 to 26 months of age, and then we calculated a frailty score in both groups. In muscle samples of 21-month old mice, we evaluated muscle quality parameters and oxidative stress markers. Finally, we performed a

transcriptomic analysis of muscle samples and highlighted differentially expressed genes in both groups of mice.

The second study was conducted to evaluate the effects of a cocktail enriched in antioxidant/anti-inflammatory molecules in a 2-month hypoactivity experiment. This countermeasure was expected to limit the effects of muscle deconditioning, but our results clearly demonstrate the ineffectiveness of this type of supplementation in the prevention of muscle mass and strength loss. Moreover, data regarding muscle molecular mechanisms highlight an alteration of recovery processes in the supplemented subjects. These results can be explained by an inhibition of the beneficial adaptations induced by the presence of RONS and illustrate the necessity of pro-oxidant molecules during long-term inactivity to maintain a certain level of muscle function. It underlines the complexity of redox balance mechanisms and demonstrates that physiological amounts of RONS are essential to activate molecular pathways and preserve positive adaptations.

Finally, the conclusions of our two studies gave clues on the suitable antioxidant modulation strategy for the prevention of skeletal muscle deconditioning. It seems preferable to focus on the stimulation of endogenous defense system whether than towards exogenous supply

of nutritional antioxidants. Nevertheless, the complexity of redox signaling requires better understanding to optimize countermeasures in muscle wasting situations.

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LIST OF ABBREVIATIONS

4E-BP1	Eukaryotic translation initiation factor 4E (eif4e)-binding protein 1
4-HNE	4-hydroxy-2-nonenal
8-OHdG	8-hydroxydésoxyguanosine
Akt	Protein kinase B
AMPK	AMP-activated protein kinase
ATF4	Activating transcription factor 4
ATGs	Autophagy-related protein
BAX	Bcl-2–associated X
BCL-2	B-cell lymphoma 2
C/EBP	CCAAT-enhancer-binding proteins
CAT	Catalase
COX IV	Cytochrome c oxidase subunit IV
CSA	Cross-sectional area
DI	Dry immersion
DNA	Deoxyribonucleic acid
eIF2α	Eukaryotic translation initiation factor 2 subunit α
eIF3F	Eukaryotic translation initiation factor 3 subunit f
FABP4	Fatty acid binding protein 4
FAPs	Fibro-adipogenic progenitors
FIP200	Focal adhesion kinase family interacting protein
Fis1	Fission 1
FOXO	Forkhead family of transcription factors
G6PD	Glucose-6-phosphate dehydrogenase
G6P	Glucose-6-phosphate
GPX	Glutathione peroxydase

GR	Glutathione reductase
GRP75	Chaperone glucose-regulated protein 75
GSH	Glutathione
GSSG	Glutathione disulfide
HDBR	Head-down bed rest
HU	Hindlimb unloading
IFN-γ	Interferon gamma
IGF-1	Insulin-like Growth Factor-1
IMAT	Intermuscular adipose tissue
KLFs	Kruppel-like Factors
LAMP-2	Lysosomal-associated membrane protein 2
LC3	Microtubule-associated protein 1A/1B-light chain 3
MAFbx	Muscle atrophy F-box (atrogin1)
MDA	Malondialdehyde
MFN2	Mitofusin 2
mTOR	Mammalian target of rapamycin
MuRF1	Muscle RING-finger protein-1
MVC	Maximal voluntary contraction
MyHC	Myosin heavy chain
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NOX	Nadph oxydase
PBS	Phosphate buffer saline
PDGFRα	Platelet derived growth factor receptor alpha
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	Phosphoinositide 3-kinase
PINK	Pten-induced kinase 1
PPAR	Peroxisome proliferator-activated receptors
PPP	Pentose phosphate pathway

PRAS	Proline-rich akt substrate
PRX	Peroxiredoxin
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPS-6	Ribosomal protein S6
SOD	Superoxide dismutase
SREBP-1c	Sterol regulatory element-binding transcription factor 1
SS-31	Szeto-schiller-31
STAT5	Signal transducer and activator of transcription 5
TBARS	Thiobarbituric acid reactive substances
TNF-α	Tumor necrosis factor α
TSC1/2	Tuberous sclerosis proteins 1 and 2
Ulk1	Unc-51 like autophagy activating kinase 1
ULLS	Unilateral lower limb suspension

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INTRODUCTION

GENERAL INTRODUCTION

Musculoskeletal system plays a key role in organism's well-functioning and is responsible for a large variety of functions such as posture, locomotion, balance, and activities of daily life. The quality of the skeletal muscle is therefore capital to maintain quality of life and, in the long term, survival (Brown & Sewry, 2017; Exeter & Connell, 2010; Trovato, Imbesi, Conway, & Castrogiovanni, 2016). However, the increase of sedentary lifestyle on one hand, and the aging of the population on the other hand, are two phenomena in expansion since the last century that share a common trait, the loss of muscle quality and function.

Indeed, between 2015 and 2050, the proportion of people aged 60 years and older will double, reaching 22% of the worldwide population, which would amount more than 2 billion of people ("WHO | Facts about ageing," 2015). The concomitant increase of actual life expectancy triggers huge public health costs, mostly due to the treatment of pathologies or co-morbidities that usually accompany aging. Concomitantly, sedentary lifestyle is increasing, especially in developed countries, as one of the undesirable adverse effects of

technological progress. Globally, a quarter of the adult population and more than 80% of the world's adolescent population are insufficiently physically active ("GHO | By category | Prevalence of insufficient physical activity among adults - Data by World Bank income groups," n.d.; "WHO | Prevalence of insufficient physical activity," 2018). In addition, situations such as injuries, diseases or pregnancy, are causes of involuntary hypoactivity or even immobilization leading to deterioration of the skeletal muscle.

Therefore, it is essential to understand molecular mechanisms leading to muscle deconditioning, in order to elaborate pertinent countermeasures to prevent its emergence, or limit the extent of its consequences. In this context, exercise training is the most effective strategy (Cartee, Hepple, Bamman, & Zierath, 2016; Furrer & Handschin, 2019; Laurin, Reid, Lawrence, & Miller, 2019; Troosters, Gosselink, & Decramer, 2002), but its utilization can be hampered by various factors, such as lack of motivation, or physical impediment (pathological pregnancy; cardiovascular disease; forced immobilization following fractures), that preclude involvement in training programs. As a consequence, alternative therapies are needed, and nutritional interventions constitute an emerging area of investigation. More particularly, this thesis will focus on antioxidant strategies as potential protectors of skeletal muscle deconditioning. It

will be studied through two axis: one in relation with aging muscles, and the other will be related to inactive muscles due to hypoactivity.

CHAPTER 1:
SKELETAL MUSCLE DECONDITIONING

1.1. Generalities

Skeletal muscle tissue represents about 40% of our total body mass and plays a key role is postural maintenance and movements production. Skeletal muscle is characterized by its high plasticity, that is the ability of the muscle to adapt to variations induced by inactivity or working demands (Harridge, 2007). Skeletal muscle deconditioning traduces a dysregulation of muscle homeostasis and is the consequence numerous factors such as, sedentary lifestyle, aging, chronic immobilization, some pathologies (cancer, chronic obstructive bronchitis…), or microgravity environment (Degens & Alway, 2006). Muscle deconditioning is considered as primary when it is the consequence of muscle disuse (voluntary or not) such as immobilization post-injury, rest during final pregnancy, hospitalization stays, or advance in age. It is considered as secondary when it is associated to chronic diseases such as cancer, type II diabetes or myopathies (Brioche, Pagano, Py, & Chopard, 2016).

Muscle deconditioning leads to an alteration of muscle function in general, and can be appreciated by various factors: loss of muscle mass and strength, structural changes, modification of muscle composition, etc… (K M Baldwin, Haddad, Pandorf, Roy, & Edgerton, 2013; Bodine, 2013; Gillis & MacDonald, 2005). These characteristics will be described in the following sections.

1.2. Characteristics of muscle deconditioning

1.2.1. Loss of muscle strength

1.2.1.1. Loss of muscle strength related to hypoactivity

The decrease of muscle function associated with muscle deconditioning is observable through non-invasive measurements. Loss of muscle strength and power are usually evaluated to attest of the loss of function. Various studies using bedrest-induced hypoactivity demonstrated loss of lower limb muscle strength, with rates from -20% to -37%, depending on the protocol duration (20 to 90 days) (Akima et al., 2000; Alkner & Tesch, 2004; Berg, Larsson, & Tesch, 1997; Ferrando, Lane, Stuart, Davis-Street, & Wolfe, 1996; Kawakami et al., 2001). Results from spaceflight experiments (whether real or simulated), are similar to those observed with unilateral lower limb suspension protocols. For example, decreases of maximal voluntary contraction in quadriceps and triceps sural muscles have

been reported in several studies (Berg, Dudley, Haggmark, Ohlsen, & Tesch, 1991; Berg & Tesch, 1996; de Boer, Maganaris, Seynnes, Rennie, & Narici, 2007). Decline in muscle stiffness has also been described, and appear since the first days of immobilization period (Grigor'ev, Kozlovskaia, & Shenkman, 2004; Grigor'eva & Kozlovskaia, 1983; Vinogradova, Stoĭda, Mano, & Iwase, 2002).

1.2.1.2. Loss of muscle strength related to aging

Decrease in muscle strength is also a key component of age-related muscle deconditioning and is one of the three characteristic of sarcopenia, together with loss of mass and poor physical performance (Alfonso J Cruz-Jentoft & Sayer, 2019). In older people, peak strength reductions achieved -20% to -40% in lower limb muscle groups, with greater loss appearing with older age (L. Larsson, Grimby, & Karlsson, 1979; Murray, Duthie, Gambert, Sepic, & Mollinger, 1985; Young, Stokes, & Crowe, 1985). Similar results are reported in upper limb too, especially in shoulder and wrist flexors muscles (Bassey & Harries, 1993; McDonagh, White, & Davies, 1984). Furthermore, it seems that rates of decline accelerate specifically between 60 and 70 years of age (Amelie Aniansson, Hedberg, Henning, & Grimby, 1986; Frontera et al., 2000). For example, the study of Hughes et al. (2001) demonstrated declines in isokinetic strength averaged 15% per decade

from the age of 46 upwards, and highlighted greater diminutions with advanced age. Moreover, Lanza et al. (2003) evidenced that muscle power, which is the product of strength and velocity, decreased with age to an even greater extent that strength. Similar results were previously observed in old men and women (Metter, Conwit, Tobin, & Fozard, 1997).

1.2.2. Loss of muscle mass

Numerous studies in animals and humans attested of an important loss of muscle mass in situations of muscle deconditioning (K M Baldwin et al., 2013; Brioche et al., 2016; Hanson, Harrison, Young, Stodieck, & Ferguson, 2013; A. D. LeBlanc et al., 1992; A. LeBlanc et al., 1988; Manini et al., 2007; M. J. White, Davies, & Brooksby, 1984).

1.2.2.1. Loss of muscle mass related to hypoactivity

A major characteristic of muscle deconditioning induced by hypoactivity is the loss of muscle mass, which appears in the very first days of hypoactivity (Cros et al., 1999). For example, Manini et al. (2007) demonstrated a reduction of thigh and calf muscles volume of 7%, in legs of healthy young adults exposed to 4 weeks of unilateral lower limb suspension. Similar results were observed in the adductor muscle group of healthy men and women after 10 and 20 days of bedrest (Kawashima, Akima, Kuno, Gunji, & Fukunaga, 2004). After

prolonged hypoactivity period, 6 months of space flight, gastrocnemius and *soleus* muscles volume were reduced by 10 and 15%, respectively (S. Trappe et al., 2009). The phenomenon of global muscle mass loss is largely due to a reduction of muscle fiber cross-sectional area (CSA), which are the principal constituents of skeletal muscles. Numerous studies focusing on muscle deconditioning due to mechanical unloading demonstrated CSA atrophy of muscle fibers (Arentson-Lantz, English, Paddon-Jones, & Fry, 2016; Demangel et al., 2017; Edgerton et al., 1995; Fitts, Riley, & Widrick, 2000; A. LeBlanc et al., 1997; Widrick et al., 1999).

It is well established that all muscles are not similarly affected by atrophy related to inactivity. Skeletal muscles are composed of "slow" fibers rich in type I myosin heavy chain (MyHC), whose oxidative metabolism make them resistant to fatigue; and "fast" fibers constituted of type II MyHC, known to generate higher force (Saltin & Gollnick, 2011). This distinction between fiber types is based on their contractile property of "time-to-peak tension" or "twitch". Type II fibers are sub classified as type IIa (considered as "intermediary fibers"), type IIx, and IIb (this isoform is present in rodents and other species but is not expressed in humans) (Egan & Zierath, 2013). Postural muscles such as soleus or cervical erector spinae muscles, mostly composed of slow fibers, are the most affected by muscle

deconditioning related to unloading, and their MyHC are privileged targets of degradation (K M Baldwin et al., 2013). Indeed, various studies showed that type I were the most degraded MyHC in atrophy induced by spaceflight or bedrest experiments (Fitts et al., 2000; S. Trappe et al., 2009; Widrick et al., 1999). For example, Fitts et al. (2010) showed a greater loss of type I fibers diameter (-20%) and CSA (-33%), than type II fibers (-16% of diameter and -29% of CSA), in soleus muscles of crew members after a 180-days spaceflight. Similar results were found in rodents exposed to microgravity condition, resulting in a substantial atrophy of antigravity muscles, whose type I fibers appeared more marked by atrophy than type II fibers (D. L. Allen et al., 1996; Caiozzo, Baker, Herrick, Tao, & Baldwin, 1994; Ohira et al., 1992; Sandonà et al., 2012; Staron et al., 1998). For example, Hikida et al. (1997) showed that 10 days of spaceflight in rats induced an atrophy of soleus fibers twice as big in type I fibers (-23,4%) than in type II (-12,5%).

1.2.2.2. Loss of muscle mass related to aging

Muscle mass is usually maintained until the age of 50 years, before declining of about 1% per year (Mitchell et al., 2012). This decrease use to accelerate with aging, reaching a global fall of muscle mass of 40% between adulthood and death (Fleg & Lakatta, 1988; Lexell, Taylor, &

Sjöström, 1988). The drop in the number of muscle fibers (about -35% between 50 and 80 years old), as well as of their diameter, both contribute to the loss of muscle mass observed with aging (A. Aniansson, Grimby, & Hedberg, 1992; Amelie Aniansson et al., 1986; Deschenes, 2004). Every fiber type is subject to atrophy but type II fibers seems to be the most affected (R S Hikida et al., 2000; Lars Larsson, Sjödin, & Karlsson, 1978; Lexell et al., 1988). Among them, MyHC expressing IIx isoform show higher size reduction than the IIa (Amelie Aniansson et al., 1986; Coggan et al., 1992). In animals, Chai et al. (2011) observed similar results in tibialis anterior muscles, with decreases of type IIb and IIx fibers CSA in old mice *vs* young ones.

1.2.3. Myotyoplogic changes

1.2.3.1. Myotypologic changes related to hypoactivity

Muscle disuse situations also affect skeletal muscle composition in terms of distribution of each fiber type. It is well established in the literature that hypoactivity's situations induce a shift from slow to fast phenotype. It is traduced by a reduction of MyHCI fibers quantity in favor of the expression of MyHCII (K M Baldwin et al., 2013). Indeed, various animal studies focusing on microgravity-induced atrophy demonstrated a decrease in the number of slow fibers concomitant with an increase in type IIx MyHC expression (D. L. Allen et al., 1996;

Caiozzo, Haddad, Baker, & Baldwin, 1996; F Haddad, Herrick, Adams, & Baldwin, 1993). For example, the study of Stevens el al. (2004) showed in soleus muscles of rats exposed to hindlimb unloading, a slow-to-fast fiber type transition, together with an increased proportion of hybrid fibers. Similar effect was also described in human experiments. Edgerton et al. (1995) reported a diminution from 48% to 40% of type I fibers after 11 days of spaceflight, and prolonged inactivity (84 days of bedrest) reduced the percentage of MyHCI from 64% to 35% (S. Trappe et al., 2004). This last study also highlighted an increase (+16%) in the proportion of hybrid fibers, co-expressing MyHCI/IIa/IIx isoforms. Interestingly, also very short-term inactivity can induce an increase in these hybrid fibers that co-express various fast MyHC (from 0,34% to 1,30 % for the MyHCI/IIx hybrid fibers) (Demangel et al., 2017). These phenotypic changes have consequences on contractile and metabolic muscle capacities, leading to reduced oxidative capacity and a wider fatigability (Egan & Zierath, 2013; Hoppeler, 2016).

1.2.3.2. Myotypologic changes related to aging

In contrast, the myotypologic changes occurring with age-related muscle deconditioning demonstrate a distinct pattern. Literature exhibit quite equivocal findings about the relative distribution of

MyHC in aging muscles (Coggan et al., 1992; Klitgaard H, Zhou M, Schiaffino S, Betto R, Salviati G, 1990; Lexell et al., 1988; T. Trappe, 2009). Coggan et al. (1992) showed in gastrocnemius muscles that the percentage of the different MyHC isoforms did not differ between old and young subjects, although type I fibers occupied a larger percentage of total muscle area in older subjects, due to smaller type II fibers. Nonetheless, it seems that the proportion of MyHC I increase in aging muscles, to the detriment of MyHC IIa and IIx (Brioche et al., 2016; Haus, Carrithers, Trappe, & Trappe, 2007). Indeed, the study of Haus and collaborators reported +55% of type I, -17% of type IIa and -26% of type IIx fibers in old vastus lateralis muscles of old individuals *vs* young ones. Moreover, the occurrence of MyHC co-expression (I/IIa and IIa/IIx fibers) increase in aged muscles, (Klitgaard H, Zhou M, Schiaffino S, Betto R, Salviati G, 1990), which is a common parameter with of myotypologic evolution also founded in muscles of deconditioned young subjects.

1.2.4. Intermuscular adipose tissue accumulation

The loss of strength associated with muscle deconditioning is higher than the rate of atrophy, indicating that other factors are involved in muscle weakness. Indeed, a longitudinal 5-years study in aged people, showed a decrease of muscle strength 2 to 5 fold higher than

the loss of muscle mass (Delmonico et al., 2009; Seene & Kaasik, 2012). In the context of real and simulated microgravity, the review of Di Prampero & Narici (2003) also described losses of muscle strength largely superior to those of muscle mass. It emphasizes the importance of other factors such as the accumulation of fat infiltration in strength's loss (O Addison, Marcus, Lastayo, & Ryan, 2014; Brioche et al., 2016). Commonly called IMAT (InterMuscular Adipose Tissue), these infiltrations are composed by adipocytes localized under muscle epimysium, between fascicles and/or muscle fibers, and represent adipocytes clusters localized outside muscular cells (Vettor et al., 2009). IMAT should not be confounded with lipid droplets, the energy stocks situated within muscular cells.

IMAT is naturally present in humans. However, an increase of its content is harmful for the muscle well-functioning. Indeed, IMAT accumulation has been observed in various situations, all tightly linked to muscle deconditioning, such as: type II diabetes (Goodpaster et al., 2003; Goodpaster, Thaete, & Kelley, 2000b), aging (Brioche et al., 2016; Ryall, Schertzer, & Lynch, 2008; M. Y. Song et al., 2004), denervation or tendinous rupture (Dulor et al., 1998; H. M. Kim, Galatz, Lim, Havlioglu, & Thomopoulos, 2012). Nonetheless, it has been demonstrated that IMAT accumulation also occurred in young and healthy individuals after a reduction in physical activity. Results

of Manini et al. (2007) highlighted in healthy subjects an increase of 15% and 20% in thigh and calf IMAT after 4 weeks of hypoactivity induced by unilateral lower limb suspension model. Moreover, the review of Addison et al. (2014) underlined a correlation between activity levels and IMAT quantity in healthy subjects. By this way, the more sedentary someone is, the more likely is to have a higher IMAT content. An increase in IMAT development has negative repercussions on metabolism, muscular power, and mobility of the subjects. Literature also demonstrated strong correlations between IMAT levels and insulin-resistance (Goodpaster et al., 2000b). Globally, muscular function alterations induced by fat cells infiltration negatively impacts on physical performance and locomotion (O Addison et al., 2014; Goodpaster et al., 2008). The study of Tuttle et al. (2012) illustrated this phenomenon showing an inverse relation between IMAT content in calf and a speed reached in a 6 minutes walking test.

The origin and role of IMAT are not completely understood. First works in animals characterized adipogenic progenitors called FAPs (fibro-adipogenic progenitors) in the interstitial muscle space. These mesenchymal stem cells, expressing the receptor PDGFRα, are able to differentiate in fibrous or adipose tissue (Judson, Zhang, & Rossi, 2013; Penton, Thomas-Ahner, Johnson, McAllister, & Montanaro,

2013; Uezumi et al., 2011). *In vitro* and *in vivo* studies in mice demonstrated the primordial role of PDGFRα+ cells in the development and the accumulation of IMAT within muscle tissue (Boppart, De Lisio, Zou, & Huntsman, 2013; Uezumi, Fukada, Yamamoto, Takeda, & Tsuchida, 2010). The study of Uezumi et al. (2014) conducted in human allowed the identification and characterization of mesenchymal progenitors expressing PDGFRα in muscular tissue. In the case of muscle deconditioning, mechanisms leading to the possible engagement of FAPs in an adipogenic linage remain unknown. Nonetheless, it seems that modifications surrounded the vascular bed could be responsible for the engagement of various progenitors in an adipogenic linage. Subsequently, a cascade of signalization involving various transcription factors (STAT5, SREBP-1c, KLFs, PPARγ, C/EBPs) allow the growth of pre-adipocytes into mature adipose cells, to finally lead to IMAT accumulation (U. A. White & Stephens, 2010).

Table 1: Summarize of muscle deconditioning characteristics in hypoactivity and aging conditions

	Hypoactivity	Aging
Muscle strength	↘	↘
Muscle mass	rapid atrophy postural muscles especially type I fibers	atrophy especially type II fibers
Myotypologic changes (% of fiber type)	↘ of type I ↗ of type IIx and hybrid fibers	↘ of type I ↗ of type IIa and IIx
IMAT accumulation	↗	↗

1.3. Dysregulation of protein balance: molecular pathways implicated

Molecular signaling involved in the regulation of skeletal muscle mass are largely responsible for the alterations seen at the functional level. This regulation is based on protein balance which controls anabolic and catabolic pathways in order to maintain protein content and optimal function (Sandri, 2008; Schiaffino, Dyar, Ciciliot, Blaauw, & Sandri, 2013). Both synthesis and degradation protein pathways are altered in muscle deconditioning situations, and it is well established that a dysregulation of their balance contributes to muscle atrophy (Chopard, Hillock, & Jasmin, 2009; Christopher S Fry & Rasmussen, 2011; Glass, 2005; Ventadour & Attaix, 2006).

1.3.1. Protein synthesis pathway

The PI3K-Akt-mTOR (Phosphoinositide 3 Kinase - Protein Kinase B - mammalian Target Of Rapamycin) axis constitutes the main signaling pathway whose activation by resistance training or protein ingestion guarantees the maintenance or increase of muscle mass (Bodine, Stitt, et al., 2001). Briefly, the activation of PI3K, stimulated by insulin or growth factors, increases the activity of Akt (also known as Protein Kinase B) (Rommel et al., 2001). This one plays an inhibitory role on TSC1/TSC2 complex by specific phosphorylations (Manning & Cantley, 2003). The interaction of mTOR with various proteins allows the formation of mTORC1 and mTORC2 complexes, which play a central role in the signaling cascade (Laplante & Sabatini, 2012). Final targets of mTOR as 4E-BP1, S6K1 or eukaryotic initiation factors (eIF3F, eIF2α) allow ribosomal biogenesis and protein translation (Holz, Ballif, Gygi, & Blenis, 2005).

In muscle deconditioning conditions, the diminution of synthesis flux is a usually observed phenomenon (Atherton et al., 2016). For example, Glover et al. (2008) showed that 14 days of joint immobilization were responsible for the decline in post-prandial synthesis flux. Body of evidence also demonstrated that inactivity in spaceflight and bedrest situations was able to reduce muscle and

whole-body protein synthesis (Chopard, Hillock, et al., 2009; Ferrando et al., 1996; Ferrando, Paddon-Jones, & Wolfe, 2002; Stein, 1999). Animal studies using hindlimb suspension or denervation models also showed a decrease of synthesis flux after only few days of unloading (Bodine, Stitt, et al., 2001; A. V Gomes et al., 2012; Hornberger, Hunter, Kandarian, & Esser, 2001). These studies demonstrated that the downregulation of some mTOR pathway actors, such as Akt, S6K1 or eIF2-α, are implicated in muscle atrophy.

During aging, the loss of muscle mass and strength characteristic of sarcopenia is also associated with a decrease in protein synthesis. Indeed, multiple studies in rodents and humans reported lower rates of muscle protein synthesis in elderly and old rodents (P Balagopal, Rooyackers, Adey, Ades, & Nair, 1997; Christopher S Fry & Rasmussen, 2011; Fadia Haddad & Adams, 2006; Léger, Derave, De Bock, Hespel, & Russell, 2008; Paturi et al., 2010; S. Welle, Thornton, Jozefowicz, & Statt, 1993; Yarasheski, Welle, & Nair, 2002). More specifically, it has been reported a decrease in MyHC synthesis during aging, which was correlated with IGF-1 plasmatic concentrations, muscle mass, and strength (P Balagopal et al., 1997). This reduced MHC synthesis is at least due to a diminished transcription because RNA amounts of different isoforms are decreased with aging, particularly type IIa and IIx isoforms (Prabhakaran Balagopal,

Schimke, Ades, Adey, & Nair, 2001; Short et al., 2005), but it is also well established that translational efficacy is lowered in the elderly (Fadia Haddad & Adams, 2006; Prod'homme et al., 2005).

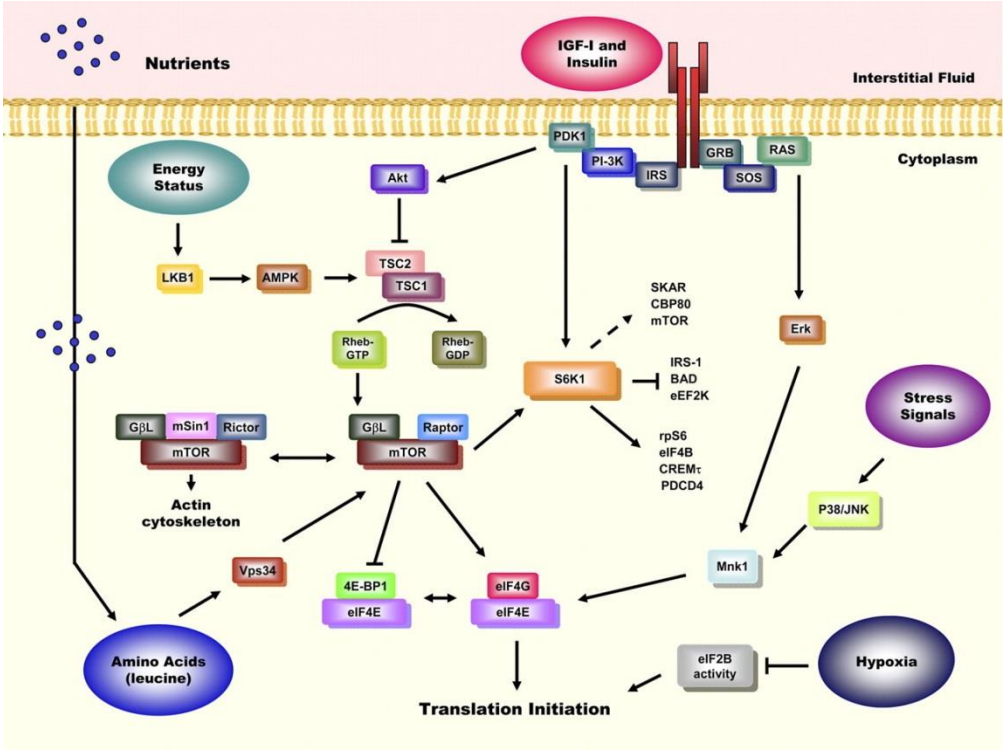

Figure 1. The main protein synthesis pathway regulated by PI3K-Akt-mTOR. Extracted from (Lang, Frost, & Vary, 2007).

1.3.2. Protein degradation pathways

There are four major pathways involved in muscle proteolysis: apoptosis, autophagy, the calpains and the ubiquitin-proteasome system. Each of these pathways and their implications in skeletal muscle deconditioning will be described in the following sections.

1.3.2.1. Apotosis

Apoptosis, also known as "programmed cell death" is a process by which cells activate their own destruction in response to stresses. It can be activated by two types of pathways: an extrinsic one involving TNF-α (tumor necrosis factor α), and an intrinsic one involving mitochondria. The intrinsic axis acts through two intracellular signaling pathways dependent or not on caspases (cysteine-dependent aspartate-cleaving proteases) (E Marzetti, Calvani, Bernabei, & Leeuwenburgh, 2012). These enzymes permit the cleavage of numerous target proteins of nuclear envelope and DNA (Danial & Korsmeyer, 2004; Dupont-Versteegden, 2005). At the end of the process, cell proceeds to its auto-destruction and resultant fragments are eliminated by macrophages (Pollack, Phaneuf, Dirks, & Leeuwenburgh, 2002).

Although research on animal models suggests a key role of apoptosis in age-related muscle loss, evidence in humans is still lacking (Dirks &

Leeuwenburgh, 2005; Dupont-Versteegden, 2005; Emanuele Marzetti & Leeuwenburgh, 2006; Siu, Pistilli, & Alway, 2005). The study of Whitman was the first to investigate age-related muscle apoptosis in humans. They reported an increase in the number of positive TUNEL cells but no changes in caspase-3/7, in vastus lateralis biopsies of older men (Whitman, Wacker, Richmond, & Godard, 2005a). Moreover, in a community-dwelling older adults, significant correlations were observed between caspase-dependent apoptotic signaling proteins and muscular thigh volume, as well as gait speed (Emanuele Marzetti et al., 2012).

Moreover, multiple studies of muscle disuse showed associations between loss of muscle CSA and the number of apoptotic cells within the tissue (Andrianjafiniony, Dupre-Aucouturier, Letexier, Couchoux, & Desplanches, 2010; Borisov & Carlson, 2000; Cheema, Herbst, McKenzie, & Aiken, 2015; Guo, Cheung, Yeung, Zhang, & Yeung, 2012; H. K. Smith, Maxwell, Martyn, & Bass, 2000). In these studies, apoptosis was measured by a variety of parameters, including caspase activation, mitochondrial EndoG release, or DNA fragmentation (involving the TUNEL staining method), and provide compelling data that apoptosis increases dramatically during the early phase of atrophy (Schwartz, 2019). Allen et al. (1997) reported for the first time an increase in apoptosis markers in atrophy induced by unloading

conditions. The authors observed an elevation of apoptotic nuclei (measured via DNA fragmentation histochemical staining) in atrophied muscles of unloaded animals ; those results were confirmed later (Siu et al., 2005). However, the experiments of Bruusgaard research's group pointed that skeletal muscle atrophy is not accompanied by myonuclear death (J. C. Bruusgaard et al., 2012; J. C. Bruusgaard, Johansen, Egner, Rana, & Gundersen, 2010; Jo C. Bruusgaard & Gundersen, 2008). Indeed, the recent review of Schwartz et al. (2019) underlined that despite the numerous studies reporting the presence of apoptotic nuclei within atrophied muscle tissue, these ones are not true myonuclei but rather condemned mononuclear cells that reside outside the muscle fiber.

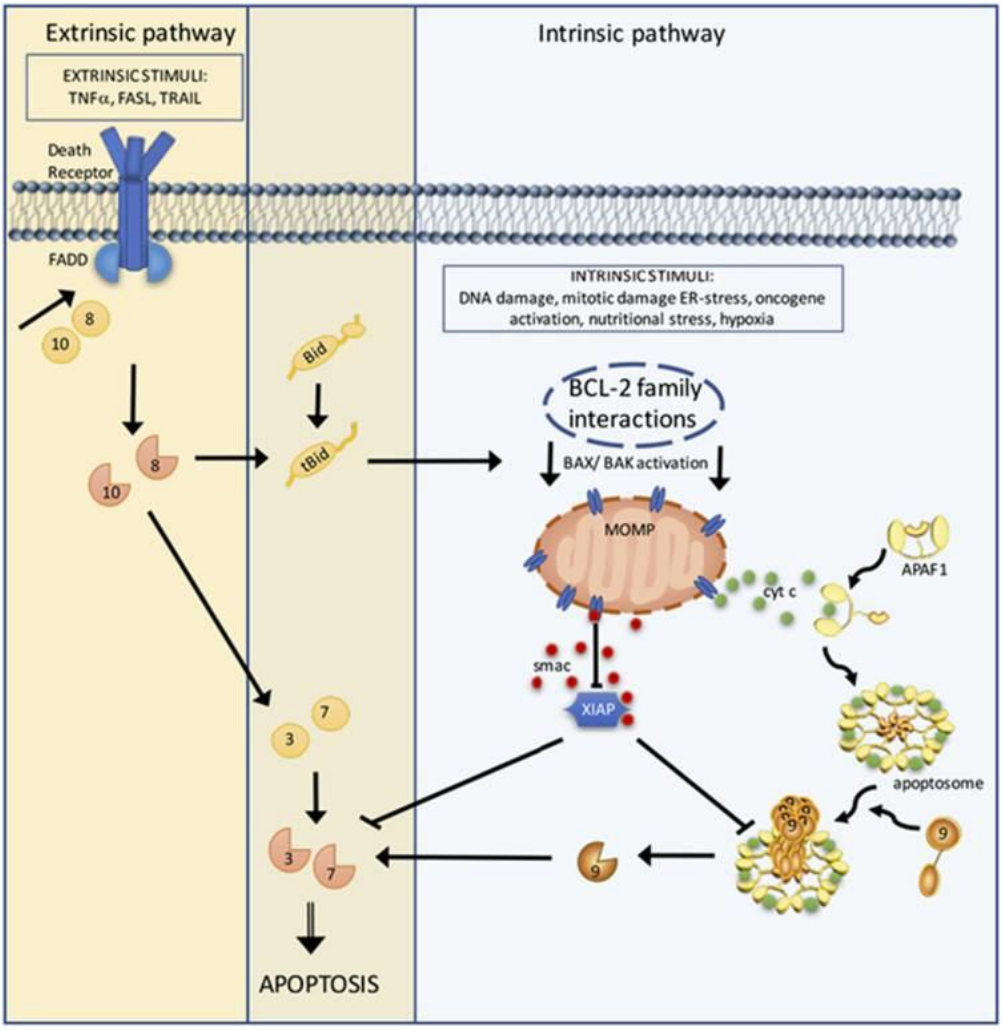

Figure 2. Apoptosis pathways : extrinsic pathway involving TNF-a and intrinsic pathway. Extracted from (Kalkavan & Green, 2018).

1.3.2.2. Calpains system

The second system is regulated by calpains, whose functioning depends on calcium concentrations (Guroff, 1964). Muscular cells contain two types of calpains (type 1 and 2, also called μ-calpain and m-calpain). Cytoskeletal and membranous proteins, enzymes and transcription factors has been identified as potential substrates for calpains (E Dargelos, Poussard, Brule, Daury, & Cottin, 2008).

Abnormal increase of calpain activity have been showed in atrophic conditions like muscle disuse or denervation (Fareed et al., 2006; Huang, Zhu, & Zhu, 2016; Matsumoto, Fujita, Arakawa, Fujino, & Miki, 2014; Nelson, Smuder, Hudson, Talbert, & Powers, 2012). Matsumoto et al (2014) found an overexpression of calpain-2 in denervated and unloaded muscles of rats. Moreover, it has been suggested that the calcium-dependent proteolytic system is involved in sarcopenia. It is based on the overall increase in calpain activities with muscle aging and supported by the idea that calpain-mediated proteolysis of myofibrillar components strongly contribute to the loss of skeletal muscle mass and function with advanced age (Elise Dargelos et al., 2007; Samengo et al., 2012).

1.3.2.3. Autophagy

The autophagic-lysosomal system is a catabolic process emerging as a major regulator of muscle mass. It recycles damaged organelles and generates metabolic substrates necessary to the maintenance of basal cellular activity (Sandri, 2010). Autophagy is the only pathway able to massively degrade macromolecules and organelles (Klionsky & Emr, 2000). It relies on the action of two vesicles, the autophagosome, which captures the substrates, and the lysosome, which, in a later step, fuse with the autophagosome and degrades it with its constituents (Levine & Klionsky, 2004). The complex Ulk1-Atg13-FIP200 plays a key role in the initiation of autophagy. Ulk1 can be phosphorylated by mTORC1, leading to its inhibition, and by AMPK, leading to its activation (Jung, Ro, Cao, Otto, & Kim, 2010). Autophagosome formation involves the action of various Atgs (autophagy related genes) and especially LC3, essential for the elongation and formation of a mature autophagosome (Nakatogawa, Ichimura, & Ohsumi, 2007).

Autophagy is essential in muscle homeostasis maintenance. A deficit of this process triggers damaged proteins and reactive oxygen/nitrogen species (RONS) accumulation in muscle, apoptosis activation, and a deterioration of intracellular structures, leading to

muscle atrophy and in some cases, muscle degeneration and myopathies (Carnio et al., 2014; Masiero & Sandri, 2010; Pal et al., 2014). On the other hand, the overactivity of the autophagic system can also conduce to amyotrophy and muscular pathologies (Mammucari et al., 2007; Sandri, 2010; Zhao et al., 2007). To summarize, both excessive and defective autophagy are highly associated with skeletal muscle loss (Petrovski & Das, 2010). Because autophagic processes promote myofibers atrophy in the young, it was thought that its inhibition would be an effective strategy to prevent sarcopenia. However, recent studies showed that the function of the autophagic lysosomal pathway actually declines during muscle aging (Zhou et al., 2017). Consistently, boosting basal autophagy would protect against aged-related muscle dysfunction by promoting the selective degradation of misfolded proteins and dysfunctional organelles (Jiao & Demontis, 2017).

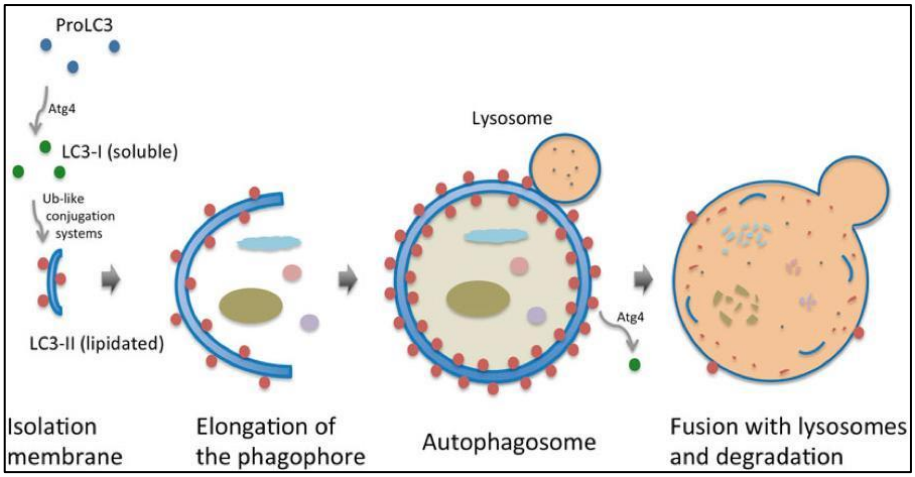

Figure 3. The autophagic-lysosomal system. Extracted from (Muñoz-Braceras & Escalante, 2016).

1.3.2.4. Ubiquitin-proteasome system

The ubiquitin-proteasome system is a protein degradation pathway playing a key role is skeletal muscle atrophy (Chopard, Hillock, et al., 2009; Lecker, Solomon, Mitch, & Goldberg, 1999). This ATP-dependent process involves the binding of the small ubiquitin protein on lysine residues of the proteins. These poly-ubiquitinated substrates are directed to the proteasome, which will be in charge of their degradation into peptides (Geng, Wenzel, & Tansey, 2012). In various situations of muscle disuse, the ubiquitin-proteasome pathway seems to be the most implicated in muscular atrophy. Indeed, numerous studies have shown that the utilization of specific inhibitors of the system had the capacity to limit the loss of muscle mass induced by

hypoactivity conditions (Caron et al., 2011; Jamart, Raymackers, Li An, Deldicque, & Francaux, 2011; Tawa, Odessey, & Goldberg, 1997).

More particularly, two genes have been identified as especially involved in atrophy in skeletal muscle: Atrogin 1 (also known as MAFbx) and MuRF1 (Muscle Ring Finger Protein 1). They code enzymes of the E3 ubiquitin ligases family, and their overexpression was observed in numerous deconditioning models with the apparition of muscle atrophy (Bodine, Latres, et al., 2001; M. D. Gomes, Lecker, Jagoe, Navon, & Goldberg, 2001). Moreover, this protein breakdown pathway is precociously activated in muscle disuse situations. Indeed, Gustafsson et al. (2010) observed an increase of mRNA levels of Atrogin1 and MuRF1 after only 3 days of unloading (unilateral lower limb suspension protocol) in humans' vastus lateralis muscles. MuRF1 knockouts mice exhibited a protection against muscle atrophy in ground-based models simulating microgravity, making of MuRF1 a promising target against loss of muscle mass (Bodine, Latres, et al., 2001; Mearini et al., 2010). However, this protection against muscle atrophy was not effective in real microgravity conditions, as discovered by Cadena et al. (2019) in their recent study, demonstrating that spaceflight induced atrophy is regulated by more complexes mechanisms.

In older muscles, some studies found an increase in MuRF1 mRNA compared to the younger ones (Dalbo, Roberts, Hassell, Brown, & Kerksick, 2011; Merritt et al., 2013; Raue, Slivka, Jemiolo, Hollon, & Trappe, 2007), while other works did not report any differences (C. S. Fry et al., 2013; Greig et al., 2011; Léger et al., 2008; Stephen Welle, Brooks, Delehanty, Needler, & Thornton, 2003; Whitman, Wacker, Richmond, & Godard, 2005b). The study of Stefanetti et al (2014) described no age-related difference in the gene expression and protein levels of Atrogin-1, MURF1, FOXO1/3, and other ubiquitin-proteasome markers, and concluded that basal muscle protein breakdown rate did not significantly differ with ageing. These results are conflicting with the previous statement supporting the idea that major age-dependent alterations in proteolysis were due to a lack of responsiveness of the ubiquitin-proteasome-dependent proteolytic pathway (Combaret et al., 2009). It traduces the complexity of protein breakdown pathways regulation in the onset of loss of muscle mass with aging.

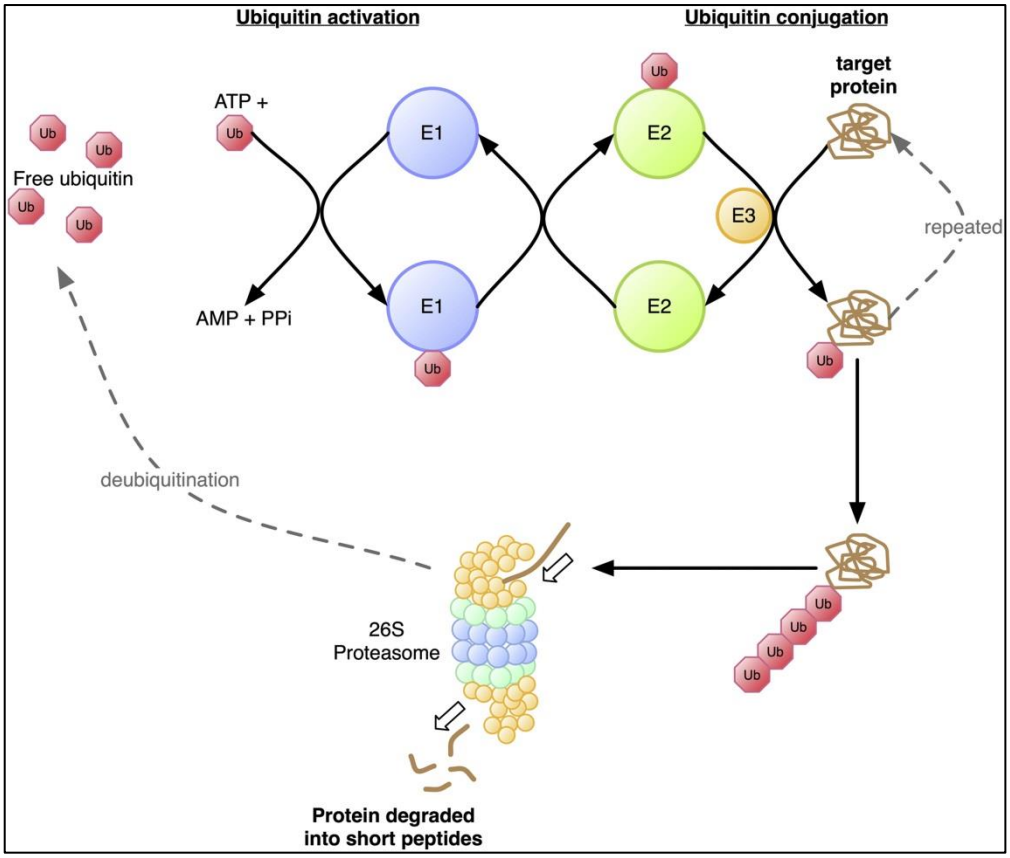

Figure 4. The ubiquitine-proteasome system. Extracted from (Murton, Constantin, & Greenhaff, 2008).

1.4. Experimental models of muscle deconditioning

In the last decades, scientific research developed various experimental models to investigate underlying processes of muscle deconditioning. These models are essential in the understanding of molecular mechanisms and the identification of potential targets, in order to test

countermeasure in the prevention of muscle wasting. This section of the thesis will attempt to describe the different experimental models of muscle disuse, from cells, to animals and finally humans' experiments.

1.4.1. Cell models of muscle wasting

To investigate molecular details of muscle atrophy, several cell culture models have been developed. Starvation of cultured cells is commonly used to induce atrophy of myotubes. In this model, cells are deprived of nutrients by replacing their culture media by PBS (phosphate buffered saline), leading to a severe atrophy (Desgeorges et al., 2014; Sandri et al., 2004; Sartorelli & Fulco, 2004; Stevenson, Koncarevic, Giresi, Jackman, & Kandarian, 2005). In such cultured myotubes undergoing atrophy, Sandri et al were the first to show a decrease in the activity of the PI3K/AKT pathway, leading to activation of Foxo transcription factors and Atrogin-1 induction (Sandri et al., 2004).

Multiple *in vitro* studies resorted to the utilization of glucocorticoids molecules, (the dexamethasone in particular), to study key mediators of muscle wasting (H. Chen et al., 2010; Schakman, Kalista, Barbe, Loumaye, & Thissen, 2013; M. G. Thompson et al., 1999). Indeed, glucocorticoids elicit muscle atrophy by increasing the rate of protein degradation via the ubiquitin-proteasome pathway and the

autophagy lysosome system (Braun & Marks, 2015). The experiments of Menconi et al. (2008) compared dexamethasone and corticosterone treatments in muscle cell lines from rats and mice. They observed a dose-dependent increase in protein degradation rates accompanied by a ~30% reduction of myotubes diameter, as well as higher Atrogin-1 mRNA levels induced by the treatments.

Finally, utilization of pro-inflammatory cytokines (such as TNF-α) in culture cells was able to induce *in vitro* atrophy, through an increase of proteolytic and apoptotic processes (Magee, Pearson, & Allen, 2008; Mirza, Pereira, Edens, & Tisdale, 2014; Moylan, Smith, Chambers, McLoughlin, & Reid, 2008). For instance, the study of Moylan confirmed that exposure of C2C12 myotubes to TNF-α triggered an upregulation of Atrogin-1 mRNA levels. The combination of TNF-α with IFN-γ, another pro-inflammatory molecule, was also used in C2C12 cell lines to stimulate muscular atrophy (Dehoux et al., 2007; Kimura et al., 2014).

It is important to mention that cultured C2C12 myotubes can also be used as a cell contraction model via their stimulation with electric pulses. It is an interesting system to test countermeasures combining exercise and nutrition, as interventions in model of cellular deconditioning (Manabe et al., 2012).

1.4.2. Animal models of muscle wasting

Rodents, predominantly rats and mice, are usually employed in the study of skeletal muscle deconditioning. Hindlimb unloading (HU), casting and denervation are the three models mainly utilized in the scientific studies focusing on muscle wasting (Bodine, Latres, et al., 2001).

1.4.2.1. Denervation

Denervation consists of the removal of nerve supply, leading to the loss of muscle contraction's capacity due to the lack of nervous stimulation. It triggers rapid deleterious effects on muscle tissue associated with a high activity of multiple proteolytic systems (Furuno, Goodman, & Goldberg, 1990). Various studies reported reductions in cross-sectional area of fibers from denervated muscles, accompanied by a loss of maximal strength (Borisov & Carlson, 2000; Carlson, Billington, & Faulkner, 1996; Dedkov, Kostrominova, Borisov, & Carlson, 2001; Schmalbruch, al-Amood, & Lewis, 1991).

1.4.2.2. Hindlimb Casting

Another model used to study muscle atrophy is the casting protocol, proposed by Frimel et al. (2005), and then frequently used to study immobilization-induced muscle wasting mechanisms. Mostly, one

hindlimb is casted to induce atrophy and the contralateral serves as control condition (Ham et al., 2015; Receno, Roffo, Mickey, DeRuisseau, & DeRuisseau, 2018; Sato et al., 2011; Vargas & Lang, 2007).

1.4.2.3. Hindlimb Unloading

In the middle of the 1970's, the NASA (National Aeronautics and Space Administration) settled the HU model, whose posterior utilization in hundreds of studies confirmed its relevance in the study of weightlessness physiological effects and muscle deconditioning (Kenneth M Baldwin, Haddad, Pandorf, Roy, & Edgerton, 2013; Globus & Morey-Holton, 2016; Morey, Sabelman, Turner, & Baylink, 1979). The HU model consists of a slight head-down inclination of the animal (about 30%), using tail or pelvic suspension. Thus, hindlimb do not reach the ground but animals are free to move, eat and groom with their forelimbs (Morey-Holton & Globus, 2002). Besides inducing a cephalad fluid shift typical of microgravity conditions, this model results in the loss of muscle mass (Chowdhury, Long, Harris, Soulsby, & Dobretsov, 2013; Cros et al., 1999; Grindeland et al., 1994; Mueller, Foley, & Hasser, 2005). Soleus muscles are especially affected by HU-induced atrophy, with a decrease in muscle forces, together with a slow-to fast transition characterized by an overexpression of the fast

MHC isoforms (Picquet & Falempin, 2003). Recently, Mortreux et al. (2018) described a brand new quadrupedal unloading model which allows the investigation of physiological alterations characteristics of partial gravity environment.

1.4.3. Human models of muscle wasting

Although cellular and animal models are useful in the understanding of muscle wasting mechanisms, the development of experimental models in humans is crucial. Three models are validated by the scientific community to study the effects of skeletal muscle inactivity in humans: the Unilateral Lower Limb Suspension (ULLS), the Head-Down Bed Rest (HDBR) and the Dry Immersion (DI).

1.4.3.1. Unilateral Lower Limb Suspension (ULLS)

Thirty years ago, Berg et al. (1991) published the first study using ULLS protocol to investigate unloading's influence on skeletal muscle. Briefly, one of the two legs is maintained in suspension thanks to an elevated sole of a shoe, which eliminates ground contact on the adjacent foot. It unloads the lower limb but allows ankle, knee, and hip joint mobility (Hackney & Ploutz-Snyder, 2012). This model has the advantage to be closely linked to clinic aspects of immobilization periods following joints or skeletal injuries for instance. Numerous studies using ULLS models confirmed the induction of muscle

deconditioning, classically characterized by loss of muscle mass, strength, and IMAT accumulation (Berg et al., 1991; Dudley, Hather, & Buchanan, 1992; Horstman, Ruiter, Duijnhoven, Hopman, & Haan, 2012; Manini et al., 2007). Contractile proteolysis rates of unloaded muscles were rapidly increased (Tesch, von Walden, Gustafsson, Linnehan, & Trappe, 2008), and fibers CSA diminished in both type I and IIa myofibers (L Brocca et al., 2015). Like the two other conditions of muscle hypoactivity that will be presented (HDBR and DI), ULLS model primarily affects soleus over *gastrocnemius* muscle fibers (Brioche et al., 2016).

1.4.3.2. Bed Rest

In the bedrest model, and more precisely the «head-down tilt bed rest» (HDBR), subjects are inclined by -6° in a supine position to induce an upward fluid shift characteristic of microgravity conditions. It is a reliable simulation model for most of the physiological effects of spaceflight, and allows the implement of countermeasure interventions (Pavy-Le Traon, Heer, Narici, Rittweger, & Vernikos, 2007). Hypoactivity induced by this model results in muscle deconditioning, and is identified by: muscle weakness and low muscle power (S. Trappe et al., 2004) ; loss of muscle mass and strength, especially in knee and ankle extensor muscles (Alkner & Tesch, 2004;

Arentson-Lantz et al., 2016; A. D. LeBlanc et al., 1992; Rittweger J1, Frost HM, Schiessl H, Ohshima H, Alkner B, Tesch P et al., 2005) ; myotypologic shift from slow-to-fast MyHC and appearance of hybrid fibers (Gallagher et al., 2005; Ohira et al., 1999; Rudnick J1, Püttmann B, Tesch PA, Alkner B, Schoser BG, Salanova M, Kirsch K, Gunga HC, Schiffl G, Lück G et al., 2004; Yamashita-Goto et al., 2001) ; reduction of myofibrillar protein content (Lars Larsson, Li, Berg, & Frontera, 1996).

1.4.3.3. Dry Immersion

In the 70's, with the growing of spatial programs, soviet researchers proposed the "dry immersion" (DI) as a brand new ground-based model of weightlessness simulation. This experimental model described for the first time by Shulzenko et al. (1976), consists in the immersion of a subject in a thermoneutral water covered with an elastic waterproof fabric, therefore the subject remains dry. It faithfully reproduce most physiological effects occurring with spaceflight: centralization of body fluids, support unloading, hypokinesia, and the lack of supporting structure under the body, which represents its considerable advantage face to the bedrest model (Navasiolava et al., 2011). DI is largely used in Russia but remains less well known elsewhere. The first experiment in Europe took place in

2015 in the space clinic of Toulouse (France). The conditions of hypokinesia and hypodynamia induced by DI reduce mechanical stress on skeletal and muscles. It leads to the fast reductions of muscle tone and tension, whose levels are superior to those observed with bedrest (Navasiolava et al., 2011). Loss of muscle mass and force are obvious after only few days of DI, and atrophy of fibers from vastus lateralis and soleus muscles have been reported in various studies (Demangel et al., 2017; Grigor'ev et al., 2004; Shenkman, Nemirovskaia, Cheglova, Belozerova, & Kozlovskaia, 1999; Shenkman et al., 2004). An increase of IMAT markers and adipogenic processes were also demonstrated in a short-term protocol of 3 days (Pagano et al., 2018), which emphasizes the effectivity of DI to rapidly induce muscle deconditioning.

Moreover, real microgravity experiments largely contribute to data collection and knowledge progress regarding muscle deconditioning mechanisms. On-board experiments on rodents (in science satellites called BION), as well as experiments in the orbital International Space Station, provide the opportunity to test new interventions (exercise/nutrition) in order to prevent deleterious effects of muscle disuse. Results of these projects are then transposable to the clinic field for chronic immobilization or forced inactivity situations (Fitts et al., 2000; S. Trappe et al., 2009).

Besides all of these models requiring expansive material and installations, an easier way to study muscle deconditioning in humans is to reduce drastically physical activity of subjects that are usually active. For example, in the study of Damiot et al. (2019), healthy and trained subjects reduced their number of steps/day from an average of 14000 to 3000.

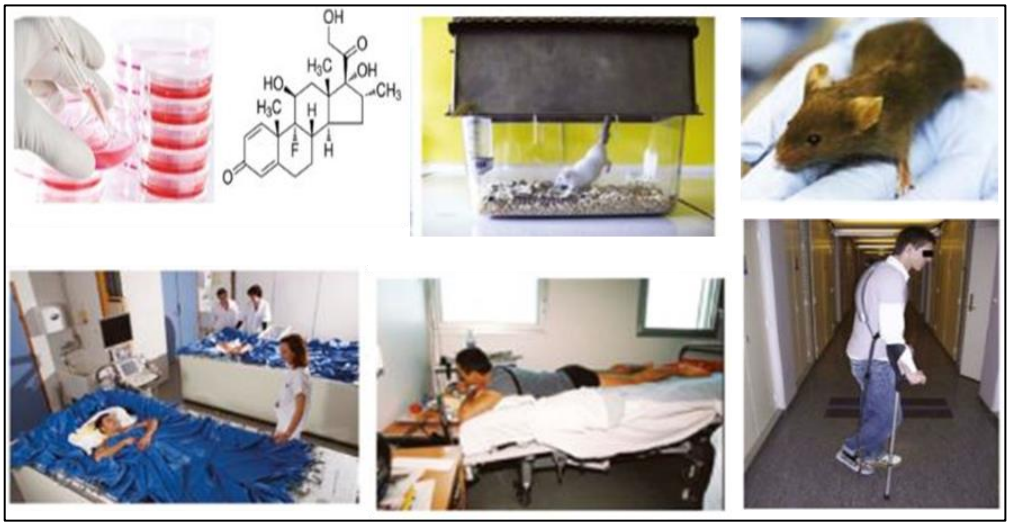

Figure 5. Experimental models of muscle deconditioning. Inspired by (Brioche et al., 2016).

1.5. Muscle deconditioning with aging

Muscle deconditioning occurring with aging is called sarcopenia. It is defined as a progressive and generalized skeletal muscle disorder that involves accelerated loss of muscle mass, and strength (Alfonso J

Cruz-Jentoft & Sayer, 2019; Fielding et al., 2011; John E. Morley et al., 2011; Muscaritoli et al., 2010). The recent review of Cruz-Jentoft & Sayer (2019) exposed the last updates of the international definition of sarcopenia. This geriatric syndrome recently considered as a pathology, is associated with an alteration of muscle function, low physical performance and reduced mobility. The aging process itself, but also physical inactivity, chronic diseases and deficient diet are all factors of its apparition (Sayer et al., 2008; D. D. Thompson, n.d.). Sarcopenia usually precedes another important age-related disorder called "frailty", whose phenotype overlaps that of sarcopenia. Indeed, sarcopenia is certainly the major cause of the onset of the physical frailty phenotype, and contributes to increase its risk of apparition (Cederholm, 2015; Landi et al., 2015).

1.5.1. The frailty syndrome

Despite the ongoing variety of concepts and definitions of frailty, recent publications agreed to consider it as a state characterized by a progressive decline of physiological systems related to aging. It results in a reduction of intrinsic capacity and confers extreme vulnerability to stressors, increasing the risk of adverse health outcomes such as low quality of life, disability, hospitalization and even death (Cesari, Landi, Vellas, Bernabei, & Marzetti, 2014; Clegg,

Young, Iliffe, Rikkert, & Rockwood, 2013; Nascimento et al., 2019; Rodriguez-Manas & Fried, 2015). Despite the large variety of aspects that covers the syndrome of frailty, there is an overall agreement to confirm that physical function plays a key role in the syndrome statement, especially in the determination of vulnerabilities status (Abellan van Kan et al., 2009; Daniels, van Rossum, de Witte, Kempen, & van den Heuvel, 2008; Ferrucci et al., 2004). Moreover, it has been evidenced that frailty is not synonymous with either comorbidity or disability. The first one is rather an etiologic risk factor whereas the second is an outcome of frailty (Fried et al., 2001). These authors established a physical phenotype of frailty, which includes the following criteria: unintentional weight loss, self-reported exhaustion, weakness (evaluated by grip strength), slow walking speed and low physical activity. Subjects were considered as "frail" if they completed three or more of these characteristics, "pre-frail" if they scored for 1 or 2 of them, and those who didn't completed any characteristics were considered "robust". Fried's criteria remain the most common way to evaluate frailty in humans; nevertheless, it is not the unique tool available. For example, Rockwood & Mitnitski (2007) proposed an individual's frailty index score based on the accumulation of deficits, which is able to quantitatively summarize vulnerability of the subjects.

Because of the changes in the epidemiological and socio-demographic profile of the population, the prevalence of frailty is increasing since the last decades, and will continue to rise in the coming years. It is estimated that frailty affects around 11% of people over 65 years old, but we know that the "pre-frail" condition -which precedes the onset of frail state- is much higher (Santos-Eggimann, Cuenoud, Spagnoli, & Junod, 2009). It is important to note that frailty is a reversible syndrome, and specific interventions can prevent its apparition (J E Morley et al., 2013). For it, it is primordial to be able to identify primary signs of occurrence's frail phenotype, to hinder its evolution and reverse the phenomenon, or at least, attenuate its adverse consequences. A prerequisite for the development of such interventions is the understanding of the molecular mechanisms leading to the onset of the syndrome (Puts et al., 2017). In that way, the development of animal models offers an interesting approach to study underlying mechanisms of frailty.

1.5.2. Animal models of frailty

The growing focus on frailty corresponds with the emergence of some animal experimental models. In addition to allow the evaluation of frailty, those tools help us to detect potential biomarkers as well as understand underlying mechanisms of frailty. Moreover, they are

essential to explore intervention strategies in order to delay its apparition.

The first experimental model was proposed by Walston et al. (2008) who worked with a genetically altered mouse (KO IL-10) who does not express the anti-inflammatory cytokine interleukin 10. This mouse model developed inflammation and exhibited strength declines consistent with those observed in frail humans, however, it cannot be considered as a model of frailty in natural aging. Later, Parks et al. (2012) developed for the first time a mouse frailty index based on 31 health-related variables including activity levels, hemodynamic measures, body composition and metabolic status. The clinical basis for the elaboration of this model was the frailty index used in humans. Although it requires invasive procedures and specialized equipment, this evaluation covers a large range of physiological systems. Another research group continued this work with the development of a mouse clinical frailty index, which regrouped 31 "clinical" items and has the advantage of being convenient, fast, and noninvasive (Whitehead et al., 2014). In 2013, Graber et al. (2013) presented the NMHSS: the mice neuromuscular healthspan scoring system, which includes functional assessments as well as in vitro muscle contractility. Its invasive procedures and time consuming measurements are the limit of this model, but it has the advantage to show a reduced individual

variability within groups. Based on the clinical phenotype model of Fried and coworkers, Liu et al. (2014) elaborated a clinically relevant frailty index for mice. It proposes the identification of four criteria of frailty (grip strength, walking speed, physical activity and endurance) and provides cut-offs to assess frailty in the evaluated animals. In a cohort of 27 to 28-months-old mice, the prevalence of frailty reached 9%, whether a value similar to that observed in human population.

All these models are described with more precision in a recent review (A E Kane et al., 2016). In 2017, the same team compared 2 frailty assessment tools in mice: the Clinical frailty index vs the Mouse frailty phenotype (Alice E Kane et al., 2017). The aim was to see whether the same mice is considered as frail with both tools and examine the association of each tool with age. The study highlights relationship between tools and potential serum markers, and concluded that both tools have value, but do not necessarily identify the same mice as frail. More recently, our research group developed a new frailty score based in the human frailty phenotype of Fried (M. C. Gomez-Cabrera et al., 2017). In this longitudinal study, mice were evaluated from 17 to 28 months old and 5 criteria were assessed: unintentional weight loss; poor endurance; slowness; weakness and motor coordination. We called it the "Valencia Score".

Besides these studies on mice models, two recent studies focused their interest on the development of frailty index in rats. Miller et al. (2017) adapted existing clinical and preclinical indices of frailty to Fisher rats. They used a battery of tasks including strength, coordination, physical activity and endurance test to classify animals as frail or mild frail. This is the first potential tool to identify frailty in old rats, and with consistence with frailty indices in mice and humans. The same year, Yorque et al. (2017) aimed to develop a rat clinical frailty index comprising 27 healthy-related deficits. The FI was scored as 0 (absent); 0.5 (mild); and or 1 (severe). They evaluated every 3-4 months a cohort of rats until their 21-months old, and showed that the FI score increased at every time point for older rats. Moreover, at the age of 17 months, a high FI score was associated with a decreased probability of survival.

Hypoactivity and aging are two situations that cause skeletal muscle deconditioning, therefore sharing common characteristics: loss of muscle strength, muscular atrophy and MyHC redistribution, as well as IMAT accumulation. To date, there is plenty of evidence supporting a causative link between oxidative stress phenomenon and muscle deconditioning; this will be described in the following part of this introduction.

CHAPTER 2:
THE REDOX BALANCE

The redox balance defines a steady state between oxidants and antioxidants molecules, essential to the well-functioning of the organism. This equilibrium is crucial to the optimal cell signaling processes that regulate various physiological functions, such as energy production, immune function, or muscle contraction (Reid, Khawli, & Moody, 1993; H. Sies, Cadenas, Symons, & Scott, 1985; H Sies, 1997; Helmut Sies, 2000).

2.1. Pro-oxidant molecules

2.1.1. Definition

Free radicals existence is known in chemistry since the beginning of the last century, and was discovered in biological systems by Commoner and collaborators 65 years ago (Commoner B, Townsend J, 1954). Soon after, Harman (1956) suggested that free radicals may account for various physiological disorders as cellular damage, mutagenesis, cancer, and degenerative processes of biological aging. Oxygen-free radicals, more generally known as Reactive Oxygen

Species (ROS), as well as Reactive Nitrogen Species (RNS), are produced by living organisms as a result of normal cellular metabolism (B Halliwell, 1987; M. Valko, Rhodes, Moncol, Izakovic, & Mazur, 2006). They play a dual role in living systems, as they can be either harmful or beneficial, depending on their quantity. Indeed, they are essential to physiological cell processes at low to moderate concentrations, but produce adverse modifications to cell components (DNA, proteins, and lipids) at higher concentrations (Barry Halliwell & Gutteridge, 2015; Marnett, 1999; Siems, Grune, & Esterbauer, 1995; Earl R Stadtman, 2004; Marian Valko et al., 2007; Wang et al., 1996). ROS and RNS can be divided into two categories, the radicals, and the non-radicals molecules (Di Meo, Reed, Venditti, & Victor, 2016). They are shown in the following table, based on Di Meo and collaborators.

Table 2: List of the main ROS and RNS

Radicals	$O_2^{\bullet -}$	Superoxide anion
	$^{\bullet}OH$	Hydroxyl
	$NO^{\bullet}$	Nitric oxide
	$NO_2^{\bullet}$	Nitrogen dioxide
Non-radicals	H_2O_2	Hydrogen peroxide
	HOCl	Hypochlorous acid
	$ONOO^-$	Peroxynitrite

2.1.2. Sources of free radicals

RONS can be generated by exogenous or endogenous sources (Finkel & Holbrook, 2000; Freeman & Crapo, 1982; Frei, 1994). Among the exogenous factors we can highlight pollution, alcohol, diet, ultraviolet light, tobacco smoke, heavy metals, industrial solvents, pesticides and radiation (Pham-Huy, He, & Pham-Huy, 2008; Phaniendra, Jestadi, & Periyasamy, 2015). On the other hand, endogenous pro-oxidant compounds come from cellular systems of the organisms where the oxygen consumption is high, such as plasma membranes, cytosols, peroxisomes and mitochondria (Di Meo et al., 2016). Most of the intracellular RONS are derived from mitochondria. The superoxide radicals are produced by complexes I and III of the electron transport chain (Finkel & Holbrook, 2000). Under normal conditions, this one is the major site of ROS production (Turrens, 1997). Peroxisomes are also involved in the formation of $O_2^{\bullet -}$, $OH^{\bullet}$, $NO^{\bullet}$ and especially H_2O_2, whose production is partly regulated by β-oxidation of fatty acids (De Duve & Baudhuin, 1966). Moreover, cytosolic enzyme systems, among others, the NADPH oxidase (NOX) family generate RONS by the NADPH-dependent one-electron reduction of oxygen to superoxide (Geiszt, Kopp, Varnai, & Leto, 2000; Inoue et al., 2003; Meitzler et al., 2014; Suh et al., 1999). The Xanthine Oxidase (XO) enzyme is also an important source of RONS because of its

implication in the production of $NO^{\bullet}$ and in the formation of $O_2^{\bullet -}$ and H_2O_2 molecules (Corte & Stirpe, 1972; D. K. Das et al., 1987).

2.2. Antioxidants

To counteract deleterious effects of an excess of RONS, organisms rely on a powerful antioxidant system. These defenses are determinant to preserve well-functioning of cells, health maintenance, and prevent the onset of chronic and degenerative diseases. Halliwell (1995) proposed a definition of an antioxidant as "a substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate". Functions of antioxidant systems are multiple. They prevent RONS formation and can block the effects of free radicals. They have the capacity to sequester reactive metabolites and so decrease their level of reactivity. They also facilitate molecular repair of radical's damage, and finally, sustain a favorable environment for the functions of others antioxidants (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012; Poljsak, Šuput, & Milisav, 2013). Antioxidant molecules are numerous, and can be classified by several ways: primary and secondary, endogenous and exogenous, enzymatic and non-enzymatic, preventative or repair-systems, hydrosoluble and liposoluble, natural or synthetic (Pisoschi & Pop, 2015).

2.2.1. Classification from a biochemical point of view

From a biochemical point of view, antioxidant molecules can be divided in two types: the enzymatic and the non-enzymatic. These two types of antioxidants are presented in the following table, based on Birben et al. (2012).

Table 3: List of enzymatic and non-enzymatic antioxidants

Enzymatic	SOD	Superoxide dismutase
	CAT	Catalase
	GPX	Glutathione peroxydase
	TRX	Thioredoxin
	PRX	Peroxyredoxin
Non-enzymatic	All trans retinol 2	Vitamin A
	Ascorbic acid	Vitamin C
	A-tocopherol	Vitamin E
	β-carotene	Carotenoids
	GSH	Glutathione

The first type prevents the production of ROS whereas the second is involved in the capture and destruction of free radicals that are generated (Sardesai, 1995).

2.2.1.1. Enzymatic antioxidants

SOD, CAT, and GPX are three key enzymes constituting the first line of antioxidant defenses. SOD are essential to dismutate superoxide anion radical $O_2^{\cdot-}$, which is perpetually generated in normal body metabolism through the mitochondrial energy production; whereas GPx and CAT are indispensable for the breakdown of H_2O_2 to harmless molecules (Ighodaro & Akinloye, 2018). SOD exists in 3 isoforms, each one dependent of a specific mineral: copper, zinc, manganese and iron (CuZn-SOD as SOD ; Mn-SOD as SOD2 and Fe-SOD as SOD3), (Fridovich, 1975; Sardesai, 1995). An adequate protection by SOD, CAT and GPx is particularly crucial for skeletal muscle functionality and protection against pathologic events (Hollander et al., 1999; Kozakowska, Pietraszek-Gremplewicz, Jozkowicz, & Dulak, 2015).

2.2.1.2. Non-enzymatic antioxidants

Examples of non-enzymatic antioxidants include endogenously synthetized molecules such as glutathione, along with agents found in diet. They are indispensable to neutralize certain types of RONS (such as •OH), against which the enzymatic defense are ineffective (Mirończuk-Chodakowska, Witkowska, & Zujko, 2018; Sardesai, 1995). Non-enzymatic antioxidants include low-molecular-weight

compounds, such as vitamins (vitamins C and E), β-carotene, uric acid, and glutathione (GSH). This one is abundant in all cell compartments and represents the main soluble antioxidant. Its production requires the presence of nicotinamide adenine dinucleotide phosphate (NADPH), whose levels are mostly determined by the glucose-6-phosphate dehydrogenase (G6PD) enzyme. GSH is a cofactor of other detoxifying enzymes, and is also involved in the conversion of Vitamin C and E to their active forms. The main antioxidant action of GSH is to give an electron to detoxify hydrogen an lipid peroxides (Birben et al., 2012; Curello et al., 1985; El-Agamey et al., 2004; Masella, Di Benedetto, Varì, Filesi, & Giovannini, 2005).

2.2.2. Classification from a cell physiology point of view

From the point of view of cell physiology, antioxidants can be classified into three categories: primary, secondary, and tertiary antioxidants (B Halliwell, 1995). The first class is involved in the prevention of new free radicals formation, and includes a wide variety of molecules, of which SOD, GR, GPx, CAT, G6PD, transferrin, ferritin and carotenoids. Secondary antioxidants represent non-enzymatic molecules, acting as RONS scavengers which suppress chain propagation reactions. They count on glutathione, flavonoids, vitamin

C and E, albumin, melatonin or uric acid. Finally, tertiary antioxidants repair oxidized biomolecules and rely on proteolytic systems to prevent their accumulation. Various types of DNA-, lipid-, and protein-repair enzymes, such as the methionine sulfoxide reductase or the phospholipase A_2, are main actors of this tertiary antioxidant class (K. J. Davies & Delsignore, 1987; Demple & Halbrook, 1983; McCall & Frei, 1999; Mehta & Gowder, 2015; Sevanian & Kim, 1985).

2.3. Concept of oxidative stress

The concept of oxidative stress was first coined by Sies (1985) as a "disturbance in the pro-oxidant to antioxidant balance in favor of the former, leading to potential damage". Oxidative stress traduces an excessive amount of RONS resulting of an imbalance between their generation and their elimination. In other words, an increase in the generation of free radicals and/or a reduction of the activity of antioxidant systems lead to oxidative stress (H Sies, 1983). Disturbances in the normal redox state lead to macromolecules damage, especially to DNA, lipids and proteins, which finally alter physiological functions and contribute to the development of numerous pathologies (Bokov, Chaudhuri, & Richardson, 2004; Cross et al., 1987). More recently, Jones et al. (2006) redefined oxidative

stress from a more "mechanistic" standpoint, and considered it as a "disruption of redox signaling".

2.3.1. Damage to DNA

DNA can be the target of various types of RONS-induced modifications: degradation of bases, mutations, translocations or deletions… (Birben et al., 2012). In nuclear and mitochondrial DNA, 8-hydroxy-2'-deoxyguanosine (8-OHdG) is one of the most common oxidative lesion induced by free radicals, and is therefore used as a biomarker for oxidative stress (Kasai & Nishimura, 1984; Valavanidis, Vlachogianni, & Fiotakis, 2009). Compared to nuclear DNA, the mitochondrial DNA is more vulnerable to oxidative damage because of its lack of histone components and of its implication in the RONS production (Richter, Park, & Ames, 1988). Moreover, mechanisms involved in the repair of DNA damage seem to be more efficient in the nucleus than in the mitochondria (Richter, Suter, & Walter, 1998; Yu, Chen, Ford, Brackley, & Glickman, 1999). Finally, high levels of RONS-induced DNA damage contribute to the progress of several diseases (cancer, neurodegeneration), but appear to be also implicated in the ageing process (Cooke, Evans, Dizaroglu, & Lunes, 2003; Evans, Dizdaroglu, & Cooke, 2004; S. P. Jackson & Bartek, 2009; Sedelnikova et al., 2004).

2.3.2. Damage to lipids

Lipids are particularly susceptible to be damaged by oxidative-derived radicals, and the process reflecting it, is known as lipid peroxidation. Since lipids are responsible for maintaining the cellular membranes integrity, extensive lipid peroxides are responsible for the alteration of their structure, composition and dynamics. More specifically, the polyunsatured fatty acids (PUFAs) are the most vulnerable target of lipid peroxidation because of their double bonds composition (Cheeseman & Slater, 1993; B Halliwell & Chirico, 1993). The products of oxidation generated by free radical attacks are multiples. The most common are the aldehydes, such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). These products can react with DNA and proteins, respectively leading to a carcinogenic effect or structural/functional damage (Frei, 1994). For a long time, he content of thiobarbituric acid reactive substances (TBARS) has been considered as a marker of lipid peroxidation; however, they are not accepted hereafter by specialized reviews because of their meagre specificity. Short-chain hydrocarbons, principally ethane and pentane are other indicators of lipids induced damage (Freeman & Crapo, 1982; B Halliwell & Cross, 1994; Janero, 1990; Rikans & Hornbrook, 1997). Finally, lipid peroxides (the endoperoxide class as the hydroperoxide class) are key mediators of

inflammation, cellular disease, and death (Gaschler & Stockwell, 2017; Ricciotti & FitzGerald, 2011).

2.3.3. Damage to proteins

Proteins are principal targets for RONS because of their overall abundance in biological systems and their implication in most of the functional processes within cells (Dalle-Donne, Rossi, Colombo, Giustarini, & Milzani, 2006; M. J. Davies, Fu, Wang, & Dean, 1999). Exposure of proteins to pro-oxidant molecules mostly induced irreparable damage, which alter protein structure and results in major deleterious consequences such as proteolysis, inhibition of enzymatic and binding activities, or alteration of immunogenicity (Dean, Fu, Stocker, & Davies, 1997; Tilman Grune, Merker, Sandig, & Davies, 2003; Ischiropoulos, 2003; Requena, Levine, & Stadtman, 2003; E. R. Stadtman & Levine, 2003). Protein oxidation alters the whole protein structure, and is characterized by alteration of amino acid side chains, cleavage of polypeptide chain and their conversion to derivates highly sensitive to proteolytic degradation (Dalle-Donne et al., 2006; Earl R. Stadtman, 2006). The hydroxyl radical $^{\bullet}OH$ is a major oxidant susceptible to attack amino acids, especially phenylalanine, methionine, tyrosine, tryptophan, cysteine, and histidine (K. J. Davies & Delsignore, 1987; E. Stadtman, 1992). One of the most common type

of ROS-induced oxidation is protein carbonylation, which refers to a process forming ketones or aldehydes, that can react with 2,4-dinitrophenylhydrazine (DNPH) to form hydrazones (E. Stadtman, 1992; Suzuki, Carini, & Butterfield, 2010). This harmful irreversible damage is considered as a major hallmark of oxidative stress-related disorders (Fedorova, Bollineni, & Hoffmann, 2014). Oxidized proteins must be degraded to limit their accumulation, but the degradation capacity of organisms loses its efficiency with aging and various disease conditions. It explains why elevated levels of oxidized proteins are found in elderly population and in number of pathological states (T Grune, Reinheckel, & Davies, 1997; E. Stadtman, 1992; Earl R. Stadtman, 2006). In addition to carbonylation damage, proteins are exposed to S-nitrosylation, which appear when cysteine residues are modified by NO (Hess, Matsumoto, Kim, Marshall, & Stamler, 2005). This type of RONS-induced modification is particularly involved in the dysfunction of ryanodine receptors, and contribute to Ca^{2+} leak and muscle weakness in aging (Andersson et al., 2011; Umanskaya et al., 2014).

2.4. The role of G6PD in oxidative stress protection

The G6PD was first characterized by Warburg & Christian in 1932, who discovered in yeast and red blood cells its implication in redox

functions. Recently, Yang et al. (2019) proposed a complete review about its implication in cell growth and cell death processes, as well as its interaction with redox signaling pathways.

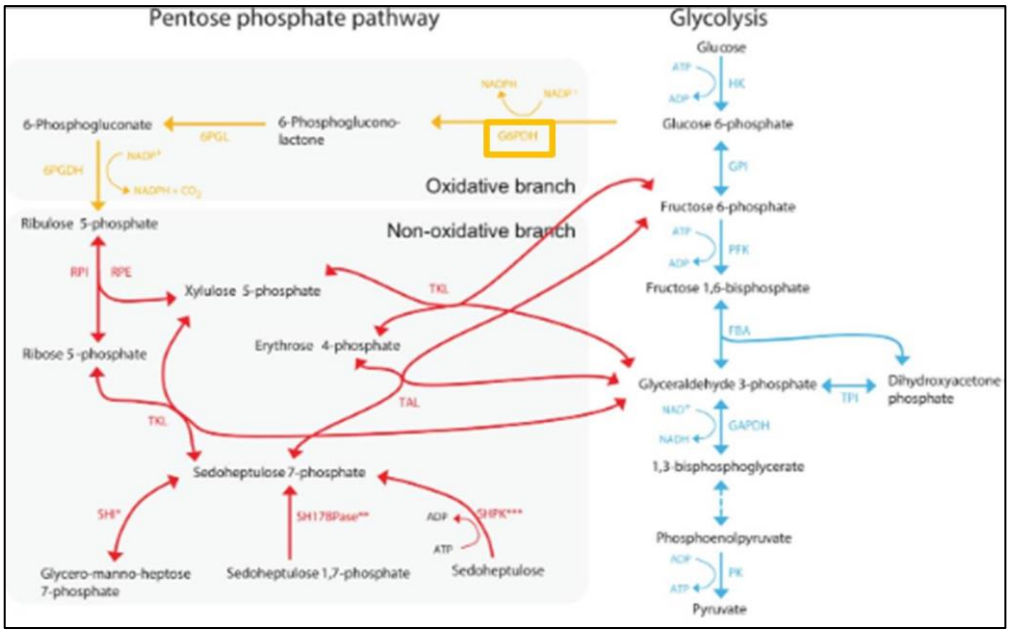

Figure 6. Schematic representation of the glycolysis and the pentose phosphate pathway (PPP), and the implication of G6PD. Extracted and modified from (Stincone et al., 2015).

2.4.1. G6PD and the glutathione antioxidant system

G6PD is a housekeeping enzyme expressed in all cells of the body, which constitutes the first and rate-limiting step of the pentose phosphate pathway (PPP), also known as hexose monophosphate

shunt. It is an alternative metabolic pathway of glycolysis, and is also involved in antioxidant defense mechanisms and biosynthetic processes (Stincone et al., 2015; Wamelink, Struys, & Jakobs, 2008). The PPP is composed of two branches: one is non-reversible and oxidative, and catalyzes the conversion of glucose-6-phosphate (G6P) to 6-phosphogluconate dehydrogenase (6PGD), generating NADPH from NADP+. The other branch is reversible and non-oxidative, and generates glycolytic intermediates and metabolites used for the synthesis of nucleic and amino acids (Hecker, Leopold, Gupte, Recchia, & Stanley, 2013).

As the main source of NADPH in cells, G6PD activity is essential to maintain adequate NADP+/NADPH ratio, whose levels are determinant for various metabolic pathways and especially antioxidant mechanisms (Y. J. Park, Choe, Sohn, & Kim, 2017; Stanton, 2012). Several antioxidant systems effectively depend on NADPH production. It is the case for mechanisms relying on SOD and CAT antioxidant enzymes, and more particularly for the glutathione system.

Indeed, NADPH is a cofactor of glutathione reductase (GR), which catalyzes the reduction of GSSG (glutathione disulfide, also called oxidized) – to GSH (the sulfhydryl form, also called reduced),

(Mannervik, 1987; Meister, 1988; Scott, Wagner, & Chiu, 1993; Helmut Sies, 1999). GSH is the central redox agent of most aerobic organisms. It doesn't need enzymatic intervention and is able to spontaneously scavenge some radical species. Its capacity to detoxify cells from free radical mostly rely on its capacity to reduce H_2O_2 in H_2O (Deponte, 2013; Pastore, Federici, Bertini, & Piemonte, 2003). The oxidation of two molecules of GSH leads to electron liberation and the consequent fusion forms one molecule of GSSG. Thereby, an increase of GSSG relative amounts leads to an alteration of the redox state of glutathione, characterized by the increase of GSSG/GSH ratio (Helmut Sies, 1986).

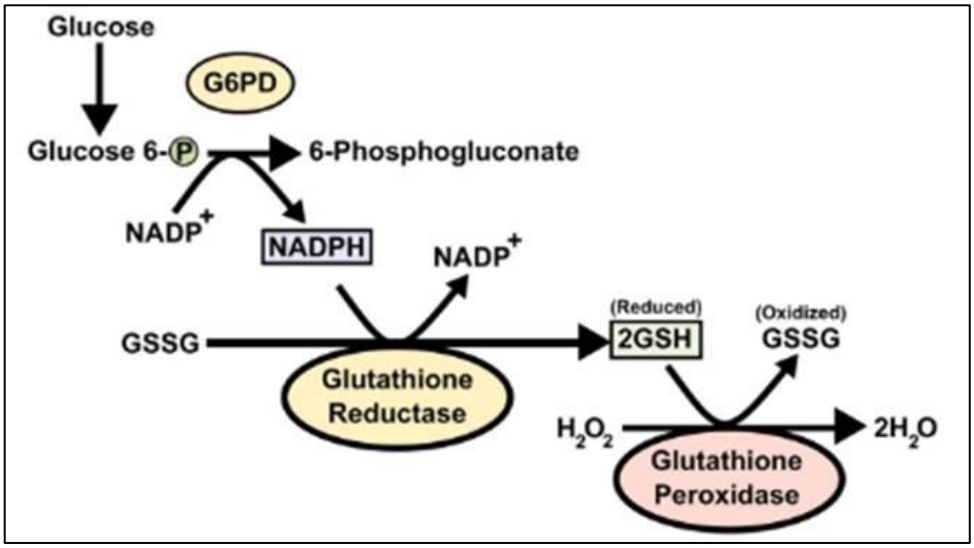

Figure 7. Implication of G6PD in the production of NADPH and its consequent role in the formation of GSH. Extracted and modified from (Moises Dominguez, 2017).

2.4.2. Modulation of G6PD activity

As explained in the previous paragraph, the role of G6PD enzyme is critical in the protection against oxidative stress. It is even more relevant in red blood cells because of their high vulnerability face to RONS (Ernest Beutler, 2008). Indeed, besides exogenous oxidant attacks, erythrocytes are exposed to endogenous free radicals generated by loading, carrying and unloading hemoglobin-bound oxygen processes. In this context, G6PD enzyme plays an essential role in supplying NADPH for the generation of GSH (Luzzatto, Nannelli, & Notaro, 2016). In 1956, it has been discovered that the hemolytic anemia was caused by G6PD deficiency (Alving, Carson, Flanagan, & Ickes, 1956). Due to the implication of G6PD in antioxidant defenses, a deficiency in this enzyme also constitutes a risk factor for the development of multiples pathologies, such as favism, diabetes, hypertension, heart failure or psychiatric disorders (Bocchetta, 2003; Hecker et al., 2013; Heymann, Cohen, & Chodick, 2012; Hwang et al., 2018).

Affecting more than 400 million people over the world, G6PD deficiency is the commonest enzyme disorder worldwide (Nkhoma, Poole, Vannappagari, Hall, & Beutler, 2009; Peters & Van Noorden, 2009). The numerous mutations (more than 300) of G6PD gene have

been classified by the World Health Organization into 5 categories, depending on clinical signs and G6PD activity levels (E Beutler, 1994; "Glucose-6-phosphate dehydrogenase deficiency. WHO Working Group.," 1989; Peters & Van Noorden, 2009). This classification is presented in the following table.

Table 4: Classification of G6PD mutations

Class	Severity mutations	% of normal G6PD function
Class I	severe	
Class II	intermediate	<10%
Class III	mild	10-60%
Class IV	asymptomatic	60-100%
Class V	activity more than normal	>150%

The numerous pathologies associated with G6PD deficiency confirm its central role in many physiological processes. Interestingly, its implication in skeletal muscle tissue maintenance has been poorly investigated. Only few studies reported some cases of rhabdomyolysis and myopathies, due to dysregulations of G6PD activity (Elias & Meijer, 1983; Kimmick & Owen, 1996; Meijer & Elias, 1984). On the other hand, some data suggest that an up-regulation of G6PD could be a good strategy to fight against sarcopenia syndrome. Indeed,

G6PD is highly regulated at transcriptional, translational and post-translational levels, and represents a downstream target of many signaling pathways (Stanton, 2012; H.-C. Yang et al., 2019). Therefore, G6PD activity and content is affected by many negative and positive regulators. Among them, insulin, growth factors and downstream molecules of the synthesis PI3K/Akt/mTOR pathway have been shown to up-regulate G6PD in muscle, leading to its hypertrophy (Kovacheva, Hikim, Shen, Sinha, & Sinha-Hikim, 2010; Max, 1984; Wagle, Jivraj, Garlock, & Stapleton, 1998).

2.5. Hypoactivity and redox regulation of muscle mass

Skeletal muscle deconditioning and especially loss of muscle mass mechanisms are influenced by RONS via a redox-mediated control of protein turnover. Indeed, several lines of evidence highlight that oxidative stress contributes to muscle atrophy, on one hand by depressing protein synthesis, and on the other hand by stimulating proteolytic processes (Powers, Morton, Ahn, & Smuder, 2016). Kondo's research group was from the first to demonstrate the contribution of redox balance dysregulation to muscle atrophy processes (Kondo, Miura, & Itokawa, 1991). In multiples animal experimentations, they investigated oxidative stress mechanisms in muscles atrophied by immobilization (Kondo, Miura, & Itokawa,

1993; Kondo, Miura, Kodama, Ahmed, & Itokawa, 1992; Kondo, Miura, Nakagaki, Sasaki, & Itokawa, 1992; Kondo, Nakagaki, Sasaki, Hori, & Itokawa, 1993). However, it is only since the last decade that the role of redox disturbances on skeletal muscle received significant attention.

2.5.1. Oxidative stress and protein synthesis pathway

The literature demonstrated that ROS production associated with disused skeletal muscles play an important role in the modulation of the protein synthesis pathway (Powers, Smuder, & Criswell, 2011). A decrease of the PI3K/Akt/mTOR protein synthesis pathway induced by oxidative stress has been demonstrated in various animal experiments. Two studies found an association between ROS oxidation products (lipid peroxidation and protein carbonylation) and IGF-1 mRNA concentrations in skeletal muscles of old rats (Clavel et al., 2006; Kovacheva et al., 2010). The authors of this last study also evidenced that an increase in Akt activation was associated with lower oxidative damage. Besides, Marzani et al. (2008) highlighted that an antioxidant supplementation in older rats restored their postprandial protein synthesis pathway. The mechanisms underlying ROS inhibition of protein synthesis are not totally understood, but some works indicated an impeding of mRNA translation at the level

of initiation process (O'Loghlen, Pérez-Morgado, Salinas, & Martín, 2006; Shenton et al., 2006). The work of Zhang et al. (2009) described in muscle cultured cells that H_2O_2 could inhibit mTOR-mediated phosphorylation of 4E-BP1 and p70S6K, two targets essential for muscle growth. Moreover, some studies revealed the role of DNA damage in the repression of mTORC1, one on the two mTOR complexes, the most involved in protein synthesis (Feng, Zhang, Levine, & Jin, 2005; Tee & Proud, 2000). Later, Tan et al. (2015) observed in myotubes of C2C12 muscle cells that chronic exposure to low levels of H_2O_2 generates oxidative stress and a decline of Akt phosphorylation, that would result in the decrease of protein synthesis. To summarize, evidence suggests that oxidative stress can depress global protein synthesis in skeletal muscle via a ROS-mediated inhibition of Akt/mTORC1 signaling. It results in slower rates of translation and contributes to the loss of muscle mass characteristic of muscle deconditioning situations. Nevertheless, antioxidant supplementation is not always an effective strategy and can on the contrary, slow down muscle hypertrophy process (Bjørnsen et al., 2016; Dutra, Alex, Silva, Brown, & Bottaro, 2019; Paulsen et al., 2014).

2.5.2. Oxidative stress and proteolytic pathways

Regarding proteolysis, whose overactivation leads to a loss of muscle mass, numerous reports reveal a critical role of RONS in the stimulation of autophagy and ubiquitin-proteasome systems.

Some data indicate the involvement of RONS in the activation of autophagy, via an inhibition of mTOR, and an increased expression of key autophagy components such as LC3, Beclin-1 and Atg4 (Kundu & Thompson, 2008; Navarro-Yepes et al., 2014; Pajares et al., 2015; Scherz-Shouval et al., 2007; Z. Yang & Klionsky, 2010). However, there is a lack of knowledge about the molecular events linking oxidative stress and autophagy in skeletal muscle tissue. In sarcopenia, many studies reported a reduction of muscle protein degradation via the autophagic pathway (C. S. Fry et al., 2013; Y. A. Kim, Kim, Oh, Kim, & Song, 2013; McMullen, Ferry, & Gamboa, 2009; O'Leary, Vainshtein, Iqbal, Ostojic, & Hood, 2013). In atrophied muscles of old rats, the increase in lipid peroxidation levels has been associated with either an upregulation of upstream autophagy regulatory proteins (Beclin-1), a downregulation of the downstream regulators (LC3 ; LAMP-2), while others have reported no changes (Atg7 ; Atg9). Therefore, it is not clear whether there is an age-associated decline in autophagic degradation (Wohlgemuth, Seo, Marzetti, Lees, & Leeuwenburgh,

2010). In inactive human skeletal muscles, a report speculated that oxidative stress would promote the expression of autophagy-related proteins (Hussain et al., 2010). Nonetheless, further research is needed to determine the relation between oxidative stress and autophagy activation in muscle disuse conditions.

Regarding protein breakdown by the ubiquitin-proteasome system, growing evidence suggests that its activation is induced by an increase of RONS in skeletal muscle tissue. *In vitro* experiments revealed that oxidative stress in myotubes (induced by H_2O_2 exposure), increased the expression of muscle specific E3 ligases, such as Atrogin-1 and MuRF1 (Li et al., 2005; Li, Chen, Li, & Reid, 2003; McClung, Judge, Talbert, & Powers, 2009). *In vivo* findings demonstrated that H2O2 generation induced by immobilization lead to an increase of E3 ligase expression in the disuse-induced atrophied muscle fibers. Moreover, a treatment with a mitochondrial-targeted antioxidant (SS-31) prevented the decline of the anabolic signaling controlled by Akt/mTOR, and was able to protect against the expression of Atrogin-1 and MuRF1, the two major atrogenes activated in muscle inactivity condition (Powers et al., 2011; Talbert et al., 2013). Similar results regarding the elevated expression of ubiquitin-proteasome effectors have been confirmed in other studies, and a higher activity of the proteasome has been correlated with loss

of muscle mass (Clavel et al., 2006; Hepple, Qin, Nakamoto, & Goto, 2008). Besides, free radicals accumulation can accelerate protein breakdown, specially of the myofibrillar proteins (MyHC, α-actinin, actin, and troponin I) in a dose-dependent manner (Tilman Grune et al., 2003; Smuder, Kavazis, Hudson, Nelson, & Powers, 2010), and therefore contribute to muscle atrophy (K. J. Davies, 1987; K. J. Davies & Delsignore, 1987). Finally, ROS accumulation stimulates protein degradation via an upregulation of key components of the ubiquitin-proteasome system, therefore contributing to muscle atrophy in aging or disuse conditions.

2.6. Redox balance and aging

As a universal, intrinsic, progressive and deleterious process, aging has been comprehensively studied and more than 300 theories have been postulated (Medvedev, 1990). Few years ago, our research group reviewed them and underlined the most prominent and well-studied theory of aging, called the free radical theory of ageing (Viña, Borrás, & Miquel, 2007). Harman (1956) hypothesized that ROS were the responsible for the damage to the cell constituents associated with aging. Although this theory enjoyed wide acceptance for several years, it has been challenged recently by many different observations (Viña, Borras, Abdelaziz, Garcia-Valles, & Gomez-Cabrera, 2013).

2.6.1. Oxidative stress in old muscles

A large body of evidence has described a dysregulation of redox balance during aging. An increase of RONS production and accumulation of oxidative damage have been reported in aged organisms, affecting especially skeletal muscle tissue (Drew et al., 2003; Sastre, Pallardó, & Viña, 2003; Vasilaki et al., 2006). High levels of oxygen consumption of skeletal muscles compared to other tissues could explain its important susceptibility to elevated RONS concentrations (Fulle et al., 2004). This accumulation of reactive species is thought to be a common determinant for muscle deconditioning in the old population, together with a compromised ability to handle elevated levels of ROS/RNS. Indeed, a significant decrease in endogenous anti-oxidant defenses in elderly could contribute to age-related muscle wasting, sarcopenia and consequently, frailty (Baumann, Kwak, Liu, & Thompson, 2016; M. J. Jackson, 2015; Kerksick & Zuhl, 2015; Scicchitano, Pelosi, Sica, & Musarò, 2018).

2.6.1.1. Oxidative damage in old muscles

Numerous studies mostly conducted on animals, demonstrated that aged muscles were sources of RONS and exhibited elevated content of reactive species (H_2O_2 ,$O_2^{\bullet-}$, $OH^{\bullet}$). In most of the cases, it was

associated with atrophy, loss of muscle weight and strength, and increased fatigability (Andersson et al., 2011; Capel, Demaison, et al., 2005; Capel, Rimbert, et al., 2005; Chabi et al., 2008; J. R. Jackson, Ryan, & Alway, 2011; Sullivan-Gunn & Lewandowski, 2013).

This overproduction of RONS in muscles is consequently traduced by an increase of macromolecule oxidative damage. Those ones are correlated with the three major characteristics current to frail subjects, that are: loss of muscle mass, strength, and walking speed (Howard, Ferrucci, & Sun, 2007; Murakami et al., 2012; Semba et al., 2007).

Many studies provide evidence for an increase of oxidative damage in aging muscles. Free radical-induced lesions to DNA, (mostly measured by 8-OHdG content, a marker of hydroxylation of bases), were increased in aging muscles of both animals (Mansouri et al., 2006; Muller et al., 2006; Ryan et al., 2008; Xu, Knutson, Carter, & Leeuwenburgh, 2008), and humans (Fanò et al., 2001; Mecocci et al., 1999). In these two studies, analysis of vastus lateralis biopsies from old subjects compared with young ones demonstrated age-dependent significant elevation of oxidative damage to DNA (measured by OH8dG), and lipids. Among the markers of lipid damage: malondialdehyde (MDA), 4-hydroxynonenal (4-HNE) and isoprostanes are usually determined. In skeletal muscle, various

studies confirmed the increase of lipid peroxidation markers content associated with sarcopenia (Barreiro et al., 2006; J.-H. Kim, Kwak, Leeuwenburgh, & Lawler, 2008; Kovacheva et al., 2010; Muller et al., 2006; Ryan, Jackson, Hao, Leonard, & Alway, 2011; Safdar, deBeer, & Tarnopolsky, 2010).

Besides, proteins are also affected by free radical damage with aging. Increase of their carbonylation levels have been observed in muscles of old rodents and humans (Barreiro et al., 2006; Clavel et al., 2006; Hepple et al., 2008; M. J. Jackson & McArdle, 2011; Muller et al., 2006; Safdar et al., 2010).

2.6.1.2. Antioxidant defenses in old muscles

The regulation of antioxidant defenses seems to be altered in the old population, contributing to an increase in oxidative stress and muscle wasting associated. However, the results regarding an alteration of antioxidant enzymes in aging muscles are quite disparate. Several studies observed a diminution of antioxidant enzymes activity and/or content (Barakat et al., 1989; Braga et al., 2008; Kovacheva et al., 2010; Kumaran, Savitha, Anusuya Devi, & Panneerselvam, 2004; Safdar et al., 2010; Senthil Kumaran, Arulmathi, Srividhya, & Kalaiselvi, 2008), while others did not find any change, or also their increase (J. R. Jackson et al., 2011; Janna R. Jackson, Ryan, Hao, & Alway, 2010; Ji,

Dillon, & Wu, 1990; J.-H. Kim et al., 2008; Ryan et al., 2008, 2011). Generally, human studies did not highlight significant alteration of the main antioxidant enzymes in muscles from old subjects (Gianni, Jan, Douglas, Stuart, & Tarnopolsky, 2004; Marzani, Felzani, Bellomo, Vecchiet, & Marzatico, 2005; Pansarasa, Bertorelli, Vecchiet, Felzani, & Marzatico, 1999).

The overall of the previously mentioned studies mostly measured on CAT, SOD and GPX enzymes, but G6PD enzyme has also been analyzed, and studies reveal a diminution of its content (Braga et al., 2008; Kovacheva et al., 2010), and activity (Barakat et al., 1989; Kumaran et al., 2004; Senthil Kumaran et al., 2008). These results highlight the critical role of G6PD in the antioxidant defenses in aging, knowing that it is an essential actor of the non-enzymatic glutathione system. Indeed, in the same works, GSH/GSSG ratio was reduced in skeletal muscle of aged animals, due to higher GSSG and lower GSH levels. These results underlined that age-related muscle wasting would be associated with an impaired glutathione system, and point the importance of G6PD enzyme as a critical endogenous defense.

2.6.2. Oxidative stress and frailty

Recently, researchers of our group proposed that oxidative stress was more associated with frailty than with aging *per se*. In a cross-sectional cohort study, men and women from 65 to 95 years of age were classified as non-frail, pre-frail and frail, according to the Fried index. The two main indicators of damage to lipids (MDA) and proteins (protein carbonylation) were analyzed in blood samples of all subjects. Results demonstrated that their levels were clearly associated to frailty, but not to age or sex (Inglés et al., 2014). From this observation, we postulated the "free radical theory of frailty". It aims at providing a rationale to delay frailty by interventions towards oxidative damage protection (Viña, 2019; Viña, Borras, & Gomez-Cabrera, 2018). As a geriatric syndrome, frailty occurs in older people, that is why the studies supporting the free radical theory of aging observed a correlation with oxidative stress markers and elderly. However, it was not the fundamental process of aging that was involved here, but the mechanisms leading to loss of muscle function that occur with the onset of frailty.

In research focusing on frailty in humans, it is important to note that muscle biopsies are very difficult to obtain, and in most of the cases, only circulating oxidative markers can be measured. Nonetheless,

various publications have found of an increase of ROS-induced damage in frail subjects. Cesari et al. (2012) showed that an increase of urinary isoprostanes (derived from lipid peroxidation) in older persons could be considered as a biomarker predicting the major adverse health-related events (mobility, disability, and death). In 2009, two studies attested of a correlation between oxidative imbalance and frailty syndrome. This status was associated with elevated GSSG, MDA and HNE concentrations. Moreover, glutathione ratio and levels of plasma protein adducts appeared as strong indices to predict frailty (Serviddio et al., 2009). In elderly Chinese population, Wu et al. (2009) underlined an association between frailty and high 8-OHdG serum levels. Recently, it has been confirmed that higher lipid peroxidation levels (detected by isoprostanes concentrations) were associated with an elevated risk of frailty (C. K. Liu et al., 2016). Finally, slow gait speed, a powerful criteria for frailty assessment, was associated with superoxide anion overproduction by NADPH Oxidase, which is one of the most important RONS provider in skeletal muscle (Baptista et al., 2012).

2.7. Antioxidant strategies against muscle deconditioning

2.7.1. Antioxidant strategies in aged muscles

Interventions targeting an increase of antioxidant defenses as a strategy to delay or reverse age-associated muscle deconditioning is an active research area(Bonetto et al., 2009; Janna R. Jackson et al., 2010; Ryan et al., 2011).

Some studies have evaluated levels of dietary antioxidants in old population and linked them with the characteristics of sarcopenia. The study of Semba et al. (2003) demonstrated in old women that higher plasma concentration in carotenoids and α-tocopherol were associated with better grip and knee strength performances. A similar report analyzed plasma vitamin C concentration and found a positive correlation with grip strength and walking speed (Saito et al., 2012). Another study highlighted the importance of dietary antioxidant levels in the development of the frailty syndrome. The authors observed that levels of vitamin E (α-tocopherol) progressively decreased from the non-frail to the frail group, and found that subjects with the highest vitamin E levels were less likely to be frail than those with the lowest concentrations (Ble et al., 2006). Recently, the work of Das et al. (2019) observed the prospective associations between antioxidant intake (analyzing vitamins A, E, C, and zinc) and frailty in older men, and concluded that poor antioxidant intake, (vitamin E in

particular), was associated with frailty. The works of Kim and collaborators underlined that sarcopenia may be prevented by a diet rich in antioxidants, especially those who come from fruit and vegetables consumption (J.-S. Kim, Wilson, & Lee, 2010; J. Kim, Lee, Kye, Chung, & Kim, 2015).

These reports suggest that antioxidant supplementation may be an efficient strategy to delay the onset of muscle deconditioning associated with ageing. However, animal or human investigations that realized such interventions have failed to demonstrate improvements in muscle mass, strength, or physical performance (or either, they didn't measure these parameters). Indeed, a 7-weeks administration of an antioxidant cocktail (vitamin E, vitamin A, zinc, and selenium) was not able to show beneficial effects on muscle mass in old animals (Marzani et al., 2008). In older people, the studies of Nalbant et al. (2009) and Bobeuf et al. (2011) failed to show an improve of muscle strength and physical performance after 6 months of supplementation with vitamin E, or a combined cocktail of vitamin C and E. All of these studies focused on an antioxidant strategy based on vitamins administration, whose action is to directly scavenge RONS, but it seems inefficient to fight against muscle deconditioning in older people.

Another antioxidant strategy would consist in providing precursors of antioxidant molecules. In animals, a long-term administration of a cystine-based antioxidant F1, (a glutathione precursor) in old mice showed beneficial effects on the prevention of loss of muscle mass associated with aging (Sinha-Hikim et al., 2013). These results support the idea that the stimulation of the endogenous antioxidant system could be a promising way to increase the defenses against RONS with aging. More research is needed in this context, especially studies evaluating the effectiveness of such interventions on the prevention of frailty. As described in the previous part of the introduction, the modulation of G6PD enzyme seem particularly relevant in this context, because NADPH is located at the core of the antioxidant defenses and the protections of the age-related oxidative damage.

2.7.2. Antioxidant strategies in inactive muscles

Antioxidant treatments have been the subject of intense research as potential countermeasure against hypoactivity-induced muscle deconditioning (Powers, 2014). Here, the efficacy of specific antioxidant supplementations in the prevention of disuse muscle wasting will be examined.

Vitamin E and its analogues have been widely explored in antioxidant interventions to prevent muscle atrophy in rodents. Kondo et al.

(1991) were the first to report a protective effect of vitamin E administration on muscle wasting induced by immobilization. Thereafter, numerous studies reported similar effects (Demiryürek S, 2004; Duarte & Soares, 1997; Kondo, Miura, Kodama, et al., 1992; Servais, Letexier, Favier, Duchamp, & Desplanches, 2007). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), an analogue of vitamin E whose antioxidant properties are characterized by the scavenging of H_2O_2 and other RONS, is known to protect against oxidative damage (Hamad, Arda, Pekmez, Karaer, & Temizkan, 2010). Its utilization as a supplement offered positive results against ventilator-induced diaphragmatic atrophy (McClung et al., 2007; Whidden et al., 2010). However, trolox administration in mice subjected to HU has not been effective against inactivity-induced limb muscle atrophy (Lorenza Brocca et al., 2010; Desaphy et al., 2010).

Resveratrol, a natural phenolic compound known for its antioxidant properties has been tested as a nutritional countermeasure to prevent deleterious effects of mechanical unloading. Fifteen days of treatment with resveratrol allowed the maintenance of protein balance, soleus muscle mass and maximal force contraction in rats subjected to the HU model (Momken et al., 2011).

Moreover, Min et al. (2011) highlighted that the administration of a novel mitochondrial-targeted antioxidant (SS-31) was able to attenuate the increase of mitochondrial ROS production due to inactivity. It also protected against oxidative stress, protease activation, and myofiber atrophy induced by 14 days of HU.

Other antioxidant compounds have been investigated as potential protectors of disuse muscle atrophy. Curcumin and N-acetylcysteine supplementations have been investigated but their administration in mice subjected to HU was not able to prevent from unloading-induced muscle atrophy and dysfunction (Farid, Reid, Li, Gerken, & Durham, 2005). In a situation of denervation, a supplementation with β-carotene attenuated the loss of muscle mass, certainly through a downregulation of key ubiquitin-proteasome elements at the early stage of atrophy processes (Ogawa et al., 2013).

Finally, combination of various antioxidants has been tested but did not always exhibit beneficial effects against hypoactivity-induced muscle deconditioning. Recently, Beytut et al. (2018) tested a cocktail supplementation with vitamin E and selenium on rats. They demonstrated its possible protective effects through a better takeover of the RONS, and an activation of the endogen defense system depending on glutathione. However, Koesterer et al. (2002) previously

experienced a treatment with a cocktail composed of vitamin E, vitamin C and β-carotene. The supplementation was able to increase the antioxidant capacity within unloaded muscles of treated animals but was ineffective against loss of muscle mass and force generation. These results underlined that combining various antioxidant compounds is a complex process which requires precaution. The interaction between different components of a cocktail, their doses, as well as the duration of the treatment, are key elements that must be taken in consideration to establish pertinent countermeasures. More research is needed to find successful and effective interventions to prevent hypoactivity-induced skeletal muscle deconditioning.

AIMS

The general aim of this PhD thesis was to evaluate the impact of the modulation of the antioxidant defenses on the prevention of muscle deconditioning. It has been studied from two perspectives, the first one in the context of aging and the second in the context of hypoactivity.

This co-supervised thesis between the universities of Valencia (Spain) and Montpellier (France) has been carried out in two different laboratories thus dealing with two different experimental projects. One project has been focused on muscle dysfunction associated with aging and has been developed in animal models. The second project deals with human hypoactivity-induced skeletal muscle deconditioning. Regarding the modulation of the antioxidant defenses in order to prevent muscle deconditioning, each project used two distinct strategies. The first one consisted of a genetic manipulation to stimulate the endogenous antioxidant defenses, while the second one investigated the effects of a nutritional exogenous antioxidants supplementation to counteract muscle wasting induced by hypoactivity.A brief context of each project is exposed, followed by a description of the material and methods employed, and finally, results are presented and discussed. A general discussion relates the most relevant points of the two studies and their implications for future interventions.

STUDY I

G6PD OVEREXPRESSION: EFFECTS ON FRAILTY IN OLD MICE

CONTEXT OF THE STUDY

In this first study, we aimed to evaluate frailty in old female animals, using WT and G6PD-overexpressing mice. We did a longitudinal functional evaluation, testing the mice each 2 months from 18 to 26 months of age, and then we calculated a frailty score in both groups. In muscle samples of 21-month old mice, we evaluated muscle quality parameters and oxidative stress markers. Finally, we performed a transcriptomic analysis of muscle samples and highlighted differentially expressed genes in both groups of mice.

MATERIAL AND METHODS

1. Experimental animals

1.1. Generation of a G6PD-transgenic mouse model

Transgenic mice were generated at the Spanish National Cancer Research Center (CNIO) at the Transgenic Mice core facility and are housed at the specific pathogen free barrier areas of the CNIO Animal House core facility. The generation process of the G6PD-Tg mice was described in a recent paper of our team (Nobrega-Pereira et al., 2016).

G6PD-Tg mouse line was generated using a 20,105 Kb human genomic DNA construct containing the entire G6PD gene, including 2.5 Kb of upstream flanking sequence and 2.0 Kb of downstream flanking sequence (Corcoran, Fraser, Martini, Luzzatto, & Mason, 1996). For transgenesis, the G6PD sequence was isolated from the pBluescript vector by NotI digestion and a 0.5 to 1 ng/µl DNA solution was injected into the pronuclei of F1 hybrids (C57BL/6J x CBA) fertilized oocytes using standard microinjection procedures. The resulting offspring was analyzed for the presence of the transgene by polymerase chain reaction (PCR) using primers specific for the human G6PD gene (Forward: 5'-AAGAAGCAGACTGGAGGAGAAG-3' and

Reverse: 5'-CAGGTTGTCACTCTCAGAACAGA-3') and that do not hybridize to the homologous mouse G6pd gene. One founder capable of transmitting the transgene to the progeny and that overexpressed G6PD was identified (+/+;tg), abbreviated here as G6PD-Tg. The founder G6PD-Tg mouse was backcrossed for three generations with pure C57BL6 mice; in this manner, all of the mice used in this study share a genetic background that is 93.75% C57BL6. All the C57BL6 mice were purchased from Harlan Laboratories and correspond to the sub-strain C57BL6/J-OlaHsd.

1.2. Animal care

After the generation of the transgenic mice in the Spanish National Cancer Research Center (CNIO), some animals were sent to the Physiology Department of the School of Medicine at the University of Valencia (Valencia, Spain) to extend the colony and perform experiments. Mice were housed in a temperature-controlled room (24±2°C) with a light-dark cycle (12:12 h). Water and food were provided ad libitum. The study was conducted following recommendations from the institutional animal care and use committee, according to the Guidelines for Ethical Care of Experimental Animals of the European Union. Experiments were always approved by the Committee on Ethics in Research of the

Medicine University of Valencia (License reference: A1444079171882). In this study, only old female mice were used, they were aged from to 18 to 26 months.

1.3. Functional tests

In order to attest the prevalence of frailty in our cohort of old mice, we evaluated 5 functional parameters: body weight, motor coordination, grip strength, running time and running speed during an incremental treadmill test. This score is based on the construct developed by Linda Fried in humans, and was called by our team the "Valencia score" of frailty (M. C. Gomez-Cabrera et al., 2017).

Body weight. Mice were weighted every 2 months using a PB3002 Delta Range balance (Mettler Scales, Toledo, OH).

Motor coordination test. The tightrope test was used to attest motor coordination capacity of mice. It is based on the method previously described by Miquel & Blasco (1978), and extensively used by our team. Mice were placed in the middle of a 60 cm long and 1,5 cm wide rope. The test was successfully passed if the mouse reached any end of the rope, or was maintained on it for 60 seconds without falling. Each mouse disposed of 3 attempts to succeed.

Maximal grip strength. To evaluate maximal grip strength of mice we used the Grip Strength Meter (Penlab, Harvard Apparatus). Mice were maintained by the tale allowing them to grasp the pull-bar of the apparatus with their forelimb for a few seconds. The peak amount of force applied was automatically registered in grams by the apparatus. The day preceding the test, mice were adapted at the apparatus. Each mouse performed 3 attempts with a minimum of 2 minutes of rest between each attempts. The best performance was noted as the maximal grip strength.

Incremental treadmill test. The animals were submitted to a graded intensity treadmill test (Model 1050 LS Exer3/6; Columbus Instruments, Columbus, OH) to determine their maximal power's aerobic metabolism. Two sessions of adaptation were processed the two anterior days before the test session. Each adaptation's session consisted in 6 minutes at 6m/min followed by 4 minutes at 8,4 m/min. The test initiated with 4 minutes at 6m/min and velocity was increased of 2m/min every 2 minutes. Exhaustion was defined as the third time a mouse could no longer keep pace with the treadmill speed and remained on the shock grid for 2 seconds rather than running. Maximal time and maximal velocity achieved at the end of the test were recorded.

1.4. The "Valencia score" of frailty

For running time, running speed and grip strength parameters, we selected at each group of age the 20% of mice which obtained the lowest performances and looked at their belonging to the WT or Tg group. We obtained a percentage of mice considered as frail in each group of mice. For the body weight criterion, we considered as frail the mice losing more than 5% of the weight they had at 18 months old. For the motor coordination parameter, the percentage of mice considered as frail represented the percentage of mice who failed the test. To elaborate the "Valencia score" of frailty summarizing the five parameters, we divided the total number of failed tests by the total number of tests performed, and we obtained the percentage of mice considered as frail in both groups.

2. Biochemical analysis

2.1. Glutathione determination

We determined GSHt and GSSG concentrations in gastrocnemius muscles using the spectrophotometric procedure described by Giutarini et al., (2013).

GSHt determination: about 25mg of muscle tissue were homogenized with a TCA 7,5E buffer (1weight/10volumes), using the Cryolys Evolution - Procellys Evolution apparatus (Bertin technologies). After

2 min of incubation at room temperature, samples were centrifuged 2min at 14000g and supernatants were collected. Then, for each sample, a cuvette was filled with the following reagents in the specified order: 945µl of PB200, 5µl of DTNB, 10µl of sample, 20µl of NADPH and 20µl of GR. The overall was mixed and absorbance was recorded at 412 nm for 1min. The same procedure was used to create a calibration curve substituting the sample by 10, 25, 50, 75 and 100 µM of GSH standards.

GSSG determination: about 30mg of muscle tissue were homogenized in 10 volumes (w/v) of a Tris-BSAN buffer (Tris, boric acid, serine, N-ethylmaleimide, pH 8). After homogenization, TCA 60% was added to the samples (10µl for 10mg) and supernatants were collected after a centrifuge of 2min at 14000g. Next, DCM was added to remove the excess of NEM prior to enzymatic determination, and supernatants were collected. For each sample, a cuvette was filled with specified order: 905µl of PB200, 5µl of DTNB, 20µl of sample, 40µl of NADPH and 20µl of GR. The overall was mixed and absorbance was recorded at 412 nm for 1min. Immediately after it, 10µl of GSSG 50µM was quickly added to the cuvette and the absorbance was recorded at the same wavelength for 1 additional minute.

2.2. Lipid peroxidation determination by HPLC

Lipids peroxidation determination as malondialdehyde (MDA) in gastrocnemius samples were performed as described by Wong et al. (1987). This method is based on the hydrolysis of lipid peroxides and subsequent formation of the adduct thiobarbituric acid (TBA) and MDA (TBA–MDA2). This adduct is detected by reverse phase HPLC and quantified at 532 nm (Ultimate 3000 Dionek). The chromatographic technique was performed in isocratic mobile phase being a mixture of 50 mM KH2PO4 (pH 6.8) and acetonitrile (70:30).

2.3. Carbonylated proteins determination

Determination of carbonylated protein levels were assessed by immunoblot detection of protein carbonyl groups using the 'OxyBlot' protein oxidation kit (Millipore, MA, USA). About 25 μg of total protein was loaded onto gels and electrophoretically separated. Antibody anti-dinitrophenylhydrazone was purchased from Intergen Company (Purchase, NY, USA). Total protein carbonyls were quantified with the OxyBlot kit by densitometry of the blotting, relativized by the densitometry of the ponceau red staining of the membrane. Specific proteins were visualized using the enhanced chemiluminescence procedure as specified by the manufacturer

(Amersham), and signals were assessed using a BioRad scanning densitometer.

2.4. Western blotting

About 30µg of gastrocnemius samples were homogenized in 10 volumes (w/v) of lysis buffer (Tris-HCl 10mM pH 7.5, 0.25M sucrose, 50mM NaCl, 5mM EDTA, 30mM sodium pyrophosphate, 1% Nonidet-P40, 0.25% sodium deoxycholate, 50mM NaF, 100µM sodium orthovanadate and 5µl of protease inhibitor cocktail per ml of buffer) and centrifuged at 12000g for 12 min at 4 °C. The protein content of the supernatant was determined by the Bradford method (Bradford, 1976). Sixty micrograms of protein extract were loaded into Stain-Free 4-20% precast gels (4568095; Bio-Rad) before electrophoretic migration and transfer onto nitrocellulose membranes (Bio-Rad; Trans-Blot Turbo Blotting System; program 7min). Then, the membranes were blocked with 1X TBST - 5% milk or BSA, and incubated overnight at 4°C with primary antibodies (see the specific section for the list of antibodies). The following day, after 3 washes of 10 min with TBST, membranes were incubated for 1h with a peroxidase-conjugated secondary antibody. The immunoblots were revealed using a Pierce ECL kit (32106; Thermo Scientific), and proteins were visualized by enhanced chemiluminescence using the ChemiDoc Touch Imaging

System and quantified with Image Lab™ Touch Software (version 5.2.1). Stain-Free technology was used as the loading control. A large number of methodological studies have already validated this technology (Vigelsø et al., 2015).

2.5. Antibodies

Primary antibodies	Reference	Commercial	Dilution
FABP4	3544S	Cell signaling	1:500
COX IV	69360	Santa-cruz	1:500
GRP75	35935S	NEB	1:1000
BAX	7480	Santa-cruz	1:200
BCL2	492	Cell signaling	1:500
4-HNE	46545	Abcam	1:1000
Secondary antibodies			
Anti-rabbit	NA934V	GE Healthcare	1:4000
Anti-mouse	NA931V	GE Healthcare	1:4000
Anti-goat	2953	Santa-cruz	1:4000

3. Histological analysis

Gastrocnemius of very old mice (34 months old) were previously included in optimal cutting temperature compound (OCT) and frozen

at -80°. Transverse serial cross sections (10 μm thick) were performed using a cryostat maintained at −25°C (HM-560, Microm H), and mounted onto glass microscope slides. Sections were colored with Hematoxylin Eosin solution and photographed with Axiovison software. The totality of muscle fibers' samples was analyzed with Image J software.

4. RNA extraction and whole transcript analysis

Total RNA from skeletal muscle was extracted using the TRIzol reagent. RNA integrity number (RIN) was tested by the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and the RNA concentration was determined by measuring absorbance at 260 nm using a spectrophotometer (GeneQuant, GE Healthcare Biosciences). In the present study, 10 microarrays were developed and analyzed: 5 from WT mice and 5 from G6PD-Tg. The synthesis of cDNA and cRNA, labeling, hybridization, and scanning of the samples were performed according to the WT Plus Reagent Kit Manual (Thermo Fisher Scientific). To prepare the hybridization cocktail, 5.5 μg of fragmented biotinylated cRNA were used. Subsequently, this cocktail was hybridized into the Clariom S Mouse microarray for 16 h at 45 °C (Thermo Fisher Scientific). This microarray contains more of 20,000 well-annotated genes. The raw

data was imported into Partek Genomics Suite v6.6 (Partek, Inc., St. Louis, MO) as CEL files. It was pre-processed, which included background correction, normalization, and summarization using robust multi-array average (RMA) analysis and then log2-transformed.

To determine global differences in genetic profiles between groups, Principal Component Analysis (PCA) and unsupervised hierarchical clustering using the Partek software was performed. The PCA is a technique used to find the causes of variability in an overall data set and ordinate them by importance. By this way, the whole set of original variables is converted into a new set of uncorrelated variables, called principal components. One-way ANOVA was used to detect differentially expressed genes using a p-value <0.05. Finally, the selected differentially expressed genes were imported into Pathway Studio version 10 (Elsevier Inc, Rockville, MD) to identify the metabolic pathway and biological processes between G6PD-Tg and WT mice.

5. Statistical analysis

Normal distribution of the samples was assessed by the Shapiro-Wilk test. To compare two different groups, the Student t-test was used, or the Mann-Whitney test in case of a non-normal distribution. For the

frailty score and its criteria, differences were tested using the Pearson's chi-squared test. Statistical analysis was made using Statistica or GraphPad Prism softwares, with a significance level set at $p<0.05$, and all graphs were represented with GraphPad Prism5 Software.

RESULTS AND DISCUSSION

1. Longitudinal evaluation of frailty

We established a frailty score based on the evaluation of 5 parameters: body weight, grip strength, motor coordination, running time and running speed achieved during an incremental treadmill test. Brut data and number of animals are available in annex tables. Here, we exposed the percentage of mice considered as frail, firstly for each of the 5 parameters, and secondly calculating the resulting "Valencia score" of frailty.

1.1. Determination of frail mice for 5 functional parameters

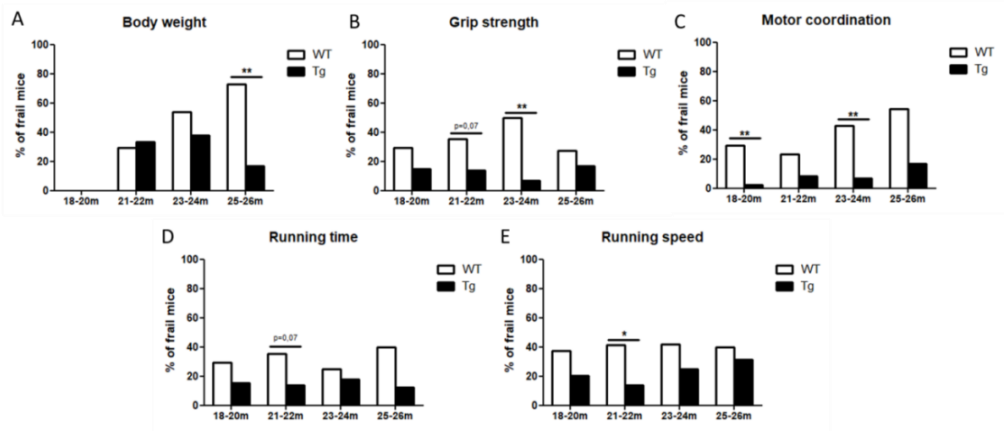

Figure 8. Percentage of mice considered as frail for each parameters: Percentage of frail mice for (A) body weight, (B) grip strength, (C) motor coordination, (D)

running time and (E) running speed, in WT and G6PD-Tg groups. A Chi²-Pearson test was using for statistical analysis, *p<0,05 ; **p<0,01.

Body weight parameter reflects animals' loss of weight with aging. We used body weight at 18 months old as the reference and calculated the percentage of loss in the following ages. An animal was considered as frail for this criterion when at any given age, it had lost more than 5% of its weight at the age of 18 months. The percentage of frail mice for body weight progressively increased in the WT group, whereas G6PD-Tg mice maintain better their weight with advancing age. At 25-26 months of age, 72% of mice were considered as frail for this criterion, compared to only 17% in the Tg group (Figure 9A).

Maximal grip strength evaluation showed that G6PD-Tg mice were stronger than WT. From 18 to 24 months old, G6PD-Tg mice exhibited better scores (strength/body weight) compared to the WT (see Table XX). At the age of 23-24 months old specifically, half of the WT mice were frail vs only 6,8% in the Tg group (Figure 9B).

The tightrope test was used to evaluate motor coordination. The animals that failed the test were considered as frail for this criterion. We observed that G6PD-Tg animals performed better than their WT congeners. For example, at 23-24 months old, 43% of the WT mice were frail for this parameter, vs 7% of the Tg mice (Figure 9C).

Mice performed an incremental treadmill test until exhaustion to evaluate their endurance capacity. Maximal running time and speed achieved at the end of the test were recorded and used to attest frailty for these two criteria (Figure 9D, 9E). For running speed parameter, more WT mice were considered as frail than the Tg (41% vs 14%), at the age of 21-22 months old. Although no statistical significance was reached ($p<0,07$), data of running time criterion give the same idea.

1.2. The Valencia score of frailty

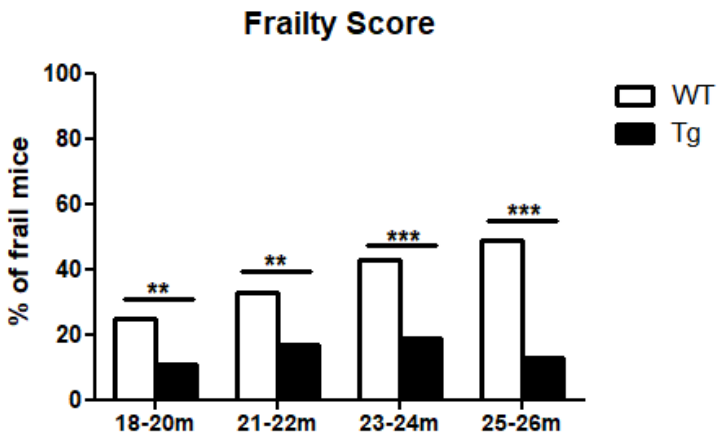

Figure 9. Percentage of mice considered as frail using the « Valencia score » of frailty. Frailty score calculation was based on the evaluation of the 5 previous parameters. A Chi²-Pearson test was using for statistical analysis *p<0.05; **p<0.01; ***p<0.001.

To see the prevalence of frailty in our cohort of old mice, we established a score of frailty based on the results obtained in each of the 5 parameters. In each group of age, we divided the total number of failed tests by the total number of tests performed, and we obtained the percentage of mice considered as frail. At each range of age (from 18 to 26 months old), the G6PD-Tg mice were less frail than the WT. As expected when studying frailty, the most convincing difference was found in the older mice (25-26 months old), where almost half of the WT mice were frail vs only 13% in the G6PD-Tg group (Figure 10).

We decided to focus our evaluation toward these 5 parameters basing us on the concept of frailty developed by *Fried 2001* in humans, who take in consideration the 5 following criteria: unintentional weight loss, self-reported exhaustion, weakness, and slow walking speed, and low physical activity. Various studies focusing on the diagnostic of frailty in animals used similar parameters in their evaluation (Baumann, Kwak, & Thompson, 2018; Kwak, Baumann, & Thompson, 2019; Yorke et al., 2017). The results of the recent study of Seldeen et al. (2019) in older female mice show similarities with ours, in terms of evolution of body weight and grip strength parameters. Kwak et al. (2019) also worked with a female cohort of WT mice, and observed that frailty prevalence increases with age: from 19% at 20 months, up to 66% in very old mice of 32 months old. In humans, prevalence of frailty ranges 5-10% at 60-70 years old, and reaches 26-65% in people older than 85 years old (Clegg et al., 2013; Collard, Boter, Schoevers, & Oude Voshaar, 2012; Gale, Cooper, & Sayer, 2015). The increase of frailty prevalence with aging, both in humans and rodents, suggests the existence of underlying common etiologies for frailty syndrome. Moreover, Gale et al. (2015) pointed that frailty appears more frequently in women than men (16% vs 12%). To complete our study, it would be interesting to evaluate a male cohort of mice and see if the same evolution would be observed.

To date, not one single molecular model has been shown to delay the onset of frailty, and we know that it is of major importance in clinical medicine. Our findings show that overexpressing G6PD in mice is an efficient strategy to delay frailty, and therefore these mice can be considered as a model of robustness. It is known that G6PD enzyme is tightly regulated. Indeed, Stanton et al (2012) already exposed a list of various positive and negative regulators of G6PD activity/location, and most of them can be modulated by nutritional and physiological interventions (Brioche et al., 2016). Research should be engage to test mechanisms able to activate G6PD enzyme and see their ability to delay the onset of frailty.

2. Oxidative stress parameters in skeletal muscles

G6PD enzyme is the main source of NADPH and produces it via the pentose phosphate pathway. NADPH, as a cofactor of glutathione reductase, contributes to ROS detoxification processes. Indeed, glutathione reductase is involved in the conversion of oxidized glutathione (GSSG) to its reduced form (GSH), and the ratio of both is used as an indicator of oxidative stress in cells. In muscle samples of old WT and G6PD-Tg mice (21 months old), we first analyzed glutathione levels and then determinated markers of RONS-induced damage to proteins and lipids.

2.1. Levels of glutathione in skeletal muscle

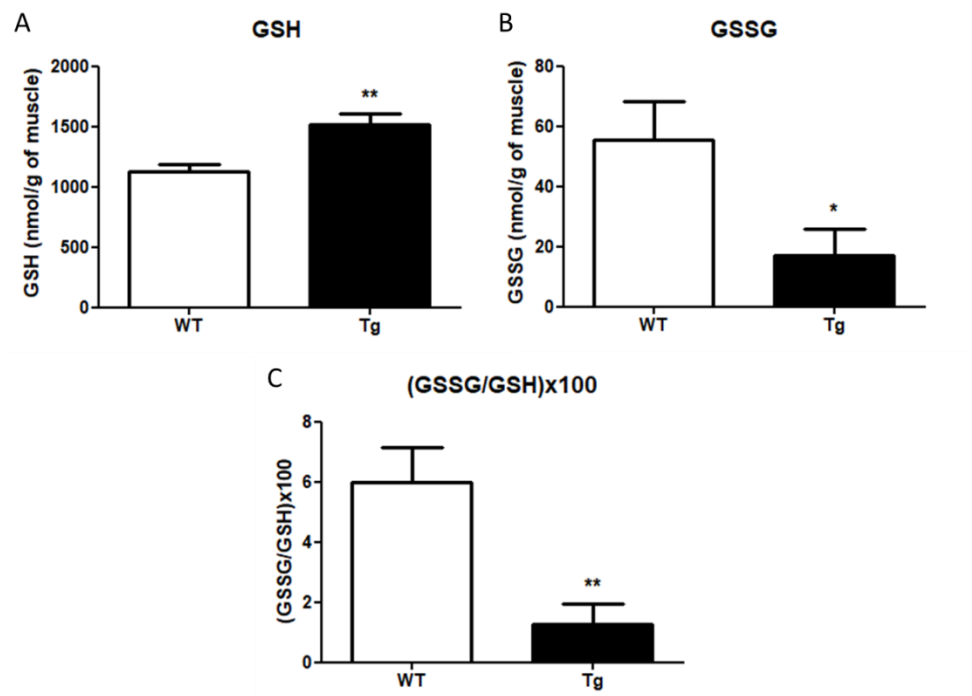

Figure 10. Levels of glutathione in gastrocnemius samples of old WT and Tg mice: (A) GSH levels (n=8 WT; n=9 Tg), (B) GSSG levels (n=6 WT; n=5 Tg) and (C) ratio GSSG/GSHx100 (n=5 WT; n=5 Tg). Bars represent means ± SEM. *p<0.05, **p<0.01.

GSH levels were higher, whereas those of GSSG were lower, in gastrocnemius samples of G6PD-Tg old mice compared to the WT. As a result, the GSSG/GSH ratio was significantly lower in the G6PD-Tg group, indicating less oxidative stress in muscles of these animals (Figure 11). These results illustrate that G6PD-Tg mice have a better capacity to reduce GSSG to GSH, thus improving the redox state of the cells.

2.2. RONS-induced damage to lipids and proteins

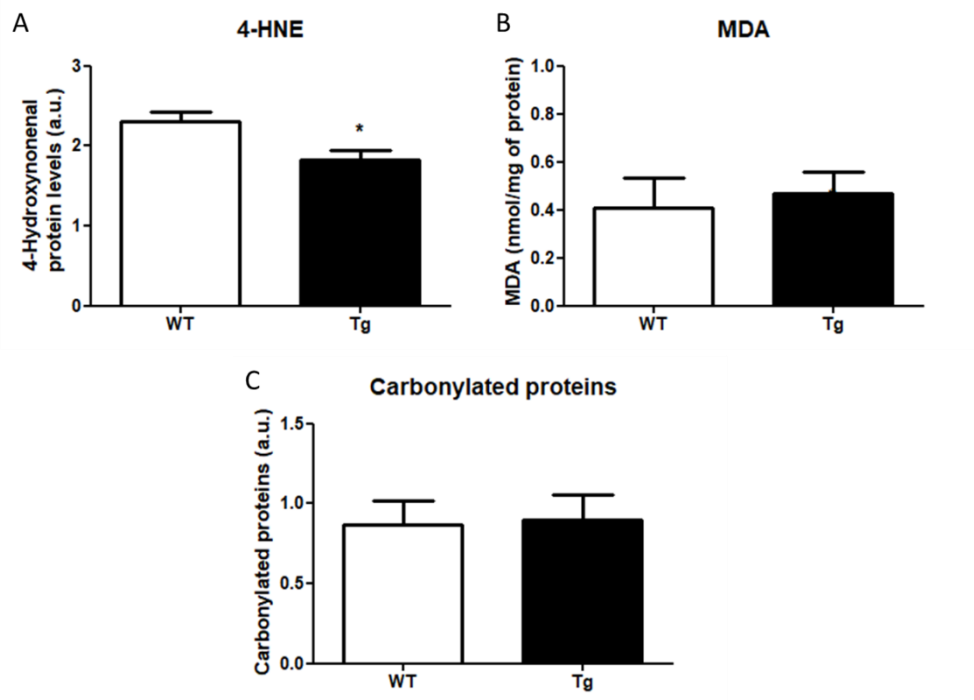

Figure 11. Markers of ROS-induced damage in gastrocnemius samples of old WT and Tg mice: (A) 4-HNE levels (n=6 WT; n=7 Tg), (B) MDA levels (n=7 WT; n=9 Tg), and (C) carbonylated proteins levels (n=8 WT; n=7 Tg). Bars represent means ± SEM. *p<0.05.

Protein carbonylation, and two products of lipid peroxidation (4-HNE and MDA), were determinated in muscle samples. We did not detect any changes in either MDA or carbonylated proteins levels between groups (Figure 12B, 12C). However, 4-HNE levels were significantly lower in G6PD-Tg muscles compared to the WT ones (Figure 12A).

In the end of the last century, Ashok & Ali (1999) recapitulated plenty of studies focusing on the role of free radicals and oxidative stress and their overall impact on the aging process. However, it was discovered later that oxidative stress was not associated with ageing itself, but specifically with frailty (Inglés et al., 2014). Indeed, this study showed in a geriatric population that circulating oxidative damage biomarkers (lipoperoxides and protein carbonylation levels), were correlated to frailty syndrome. Few years earlier, Serviddio et al. (2009) also exposed that frailty was associated with an increase of oxidative stress markers such as MDA, 4-HNE and GSSG. To understand molecular basis of frailty, an experimental model of SOD1-KO mice, lacking the antioxidant enzyme Cu/Zn superoxide dismutase has been developed (Deepa et al., 2017). These animals were considered as an excellent mouse model of frailty and presented alterations in oxidative stress pathway (increased levels of lipid peroxidation in plasma and muscle; increased H_2O_2 generation). Nevertheless, the contrary proposal, i.e., that animals with an improved redox state could be protected against frailty had not been tested. The G6PD-Tg mice that we described constitute the first molecular model to show a protection against frailty. Altogether, our present results indicate that G6PD-Tg mice beneficiate of a better protection against oxidative stress thanks to an improved glutathione system, but consequences in terms of

macromolecules damage detection were not so evident. We hypothesize that there were certainly little damage in the control animals, impeding the detection of a difference with the transgenic animals. Another possible factor is the age of the mice (21-months old) from which analyzes were realized. Muscle samples of older mice of 25-26 months old would certainly allow the detection of differences in MDA and oxidized proteins levels between our two groups.

3. Markers of skeletal muscle quality

Performance in maximal grip strength test demonstrated that G6PD-Tg mice were stronger than the WT. In this optic, we investigated molecular indicators of skeletal muscle well-functioning in gastrocnemius samples of 21-month-old mice.

3.1. Evaluation of skeletal muscle mass

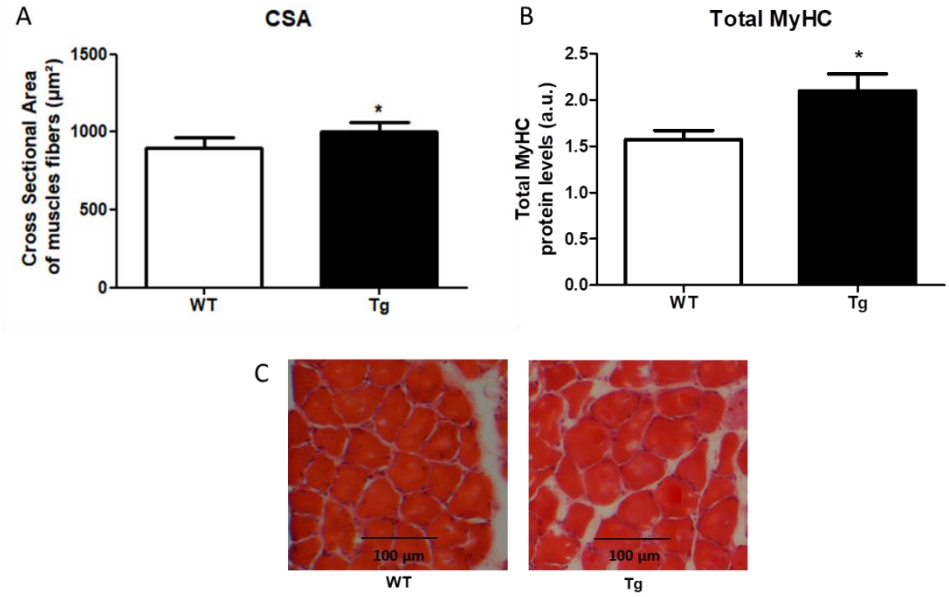

Figure 12. Evaluation of skeletal muscle mass of old WT and Tg mice: (A) Cross-sectional area measurement of muscle fibers (µm²) from 34 months old mice (n=8 WT; n=7 Tg), (B) Total myosin heavy Chain protein content in gastrocnemius samples of 21 months old mice (n=7 WT; n=6 Tg), (C) Representative images of hematoxylin-eosin coloration used to quantify CSA. Bars represent means ± SEM. *p<0.05.

We first assessed cross-sectional area (CSA) of muscle fibers of very old mice thanks to histological analyzes. Mean CSA of G6PD-Tg mice were higher than the WT mice (1005 µm² vs 901µm², respectively), (Figure 13A). Total myosin heavy chain (MyHC) protein contents in muscles support this result, as its levels were higher in G6PD-Tg muscles compared to the WT ones (Figure 13B).

3.2. Indicators of muscular "quality"

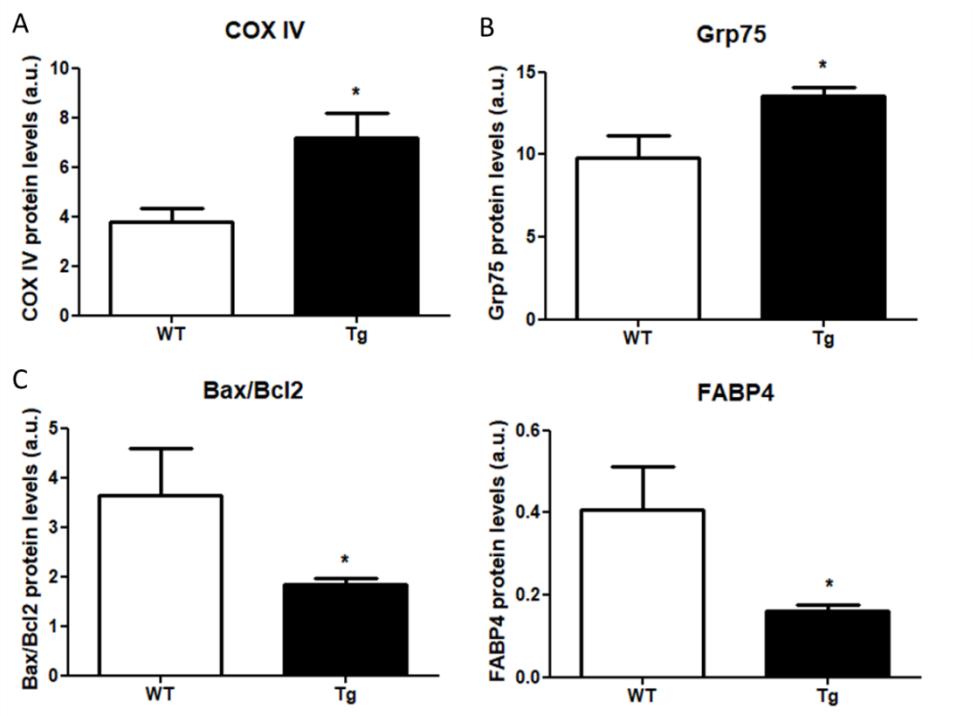

Figure 13. Muscle "quality" parameters in WT and Tg mice: (A) Protein levels of COX IV (n=7 WT; n=7 Tg), (B) Grp75 (n=7 WT; n=7 Tg), (C) Bax/Bcl2 ratio (n=7 WT; n=7 Tg) and FABP4 (n=6 WT; n=6 Tg), in gastrocnemius samples of 21 months old mice. Bars represent means ± SEM. *$p<0.05$.

We determined the expression of various markers related to muscle quality in samples of old mice. Two indicators of mitochondrial function were determined: COX IV, one of the nuclear encoded subunits of cytochrome c oxidase; and Grp75 (also known as mortalin, or mtHsp70), a mitochondrial chaperone involved in the refolding of redox-modified proteins and in mitochondrial biogenesis (Deocaris,

Kaul, & Wadhwa, 2006). Protein levels of both were significantly increased in G6PD-Tg samples compared to the WT (Figure 14A, 14B). Grp75 is also a well-known inhibitor of apoptotic processes, through the modulation of the muscle apoptosis indicator, Bax/Bcl2 ratio (Chelh et al., 2009). This marker was lower in muscles of G6PD-Tg mice than in the WT (Figure 14C). We finally evaluated the content of fatty acid binding protein 4 (FABP4), a marker of intermuscular adipose tissue. FABP4 level was diminished in G6PD-Tg mice compared to the WT, indicating a probable higher content of adipocytes in skeletal muscles of these animals (Figure 14D).

It is known for a long time that the loss of muscle mass associated with aging implicates the reduction of muscle fibers' CSA (Amelie Aniansson et al., 1986; Lexell et al., 1988). It is mostly the consequence of a loss of myofibrillar protein synthesis, especially Myosin Heavy Chain (MyHC), the most abundant protein by mass in skeletal muscles (P Balagopal et al., 1997; Cuthbertson et al., 2005). Protein contents of MyHC are reduced in muscles of old animals compared to young ones, and it is certainly due to a reduction of its transcription because mRNA amounts of different MyHC isoforms decrease during aging (Prabhakaran Balagopal et al., 2001; Fadia Haddad & Adams, 2006; L. V Thompson, Durand, Fugere, & Ferrington, 2006). Moreover, various studies in old mice demonstrated that reduced G6PD activity

and/or protein content were associated with an increase of atrophy and apoptosis (Braga et al., 2008; Kovacheva et al., 2010; Sinha-Hikim et al., 2013). Here, the higher CSA of muscle fibers from G6PD-Tg mice, as well as their higher muscular content of MyHC, attest of a protection against atrophy compared to the WT old mice.

Various studies conducted in elderly population demonstrated that aging muscles are suggested to an impairment of mitochondrial function that contributes to fiber atrophy and the decline of muscle performance (Derbré et al., 2012; Faitg, Reynaud, Leduc-Gaudet, & Gouspillou, 2017; J. Miquel, Economos, Fleming, & Johnson, 1980). The study of Joseph el al (2012) confirmed that poor physical performance in old individuals was associated with a decline in mitochondrial respiration, COX activity, as well as mtHsp70 protein content. The expression of this mitochondrial chaperone (also called Grp75) was positively correlated with increased VO2max in human skeletal muscles (Venojärvi et al., 2008). Besides being involved in mitochondrial biogenesis and oxidative stress-related survival pathways, Grp75 is implicated in apoptosis inhibition (Londono, Osorio, Gama, & Alzate, 2012). Apoptosis is a cellular process known to increase significantly with age, which likely contributes to aged-associated declines. Indeed, acceleration of this process in skeletal muscle fibers represents a key mechanism underlying sarcopenia,

which constitute, per se, a major risk factor for frailty (Dirks & Leeuwenburgh, 2005; Emanuele Marzetti & Leeuwenburgh, 2006; Whitman et al., 2005a). Indeed, many studies cited in the two reviews just mentioned, reported a correlation between sarcopenia markers and rates of apoptosis. Proteins of the Bcl-2 family are key regulators of the mitochondrial release of apoptotic mediators. Notably, Bax and Bcl-2 play an important role as pro and anti-apoptotic proteins, respectively, and their balance (Bax/Bcl2) is commonly used to evaluate apoptosis in cells (Danial & Korsmeyer, 2004). Various studies reported an increase of Bax/Bcl2 ratio in muscles of old animals (Alway, Degens, Krishnamurthy, & Smith, 2002; Chung & Ng, 2006; W. Song, Kwak, & Lawler, 2006). In our study, lower Bax/Bcl2 levels in G6PD-Tg compared to the WT animals, indicate a lower activity of apoptotic processes in the mice overexpressing the antioxidant enzyme. Moreover, cell culture studies reported a direct negative relation between G6PD activity/protein content and apoptosis (Fico et al., 2004; Nutt et al., 2005; Tian et al., 1999). Together with the markers of a higher mitochondrial content (COX IV and Grp75), our results point some improved molecular mechanisms explaining why G6PD-Tg old mice performed better than the WT in functional tests.

We also investigated the presence of intermuscular adipose tissue (IMAT) in muscles of our old mice, as another characteristic of muscle deconditioning. It has been demonstrated that IMAT accumulation constitutes an independent variable leading to sarcopenia. Indeed, fat deposition levels in skeletal muscles are known to increase with age (Brioche et al., 2016; Demangel et al., 2017; Goodpaster, Thaete, & Kelley, 2000a; Kirkland, Tchkonia, Pirtskhalava, Han, & Karagiannides, 2002). The study of Schafer et al. (2010) demonstrated a correlation between IMAT levels and the loss of muscle mass and strength in an elderly cohort. The importance of IMAT contribution to the impairment of mobility in older adults was demonstrated in the study of Marcus et al. (2012), and literature also revealed that IMAT levels were increased in frail vs non-frail individuals (Odessa Addison et al., 2014). To date, any studies conducted in animal model analyzed fat accumulation in skeletal muscle tissue in the context of frailty. Here, our results support our previous conclusions indicating a better muscle quality of the old G6PD-Tg mice compared to the WT.

4. Transcriptomic analysis in skeletal muscle samples

4.1. Changes in transcriptomic profile

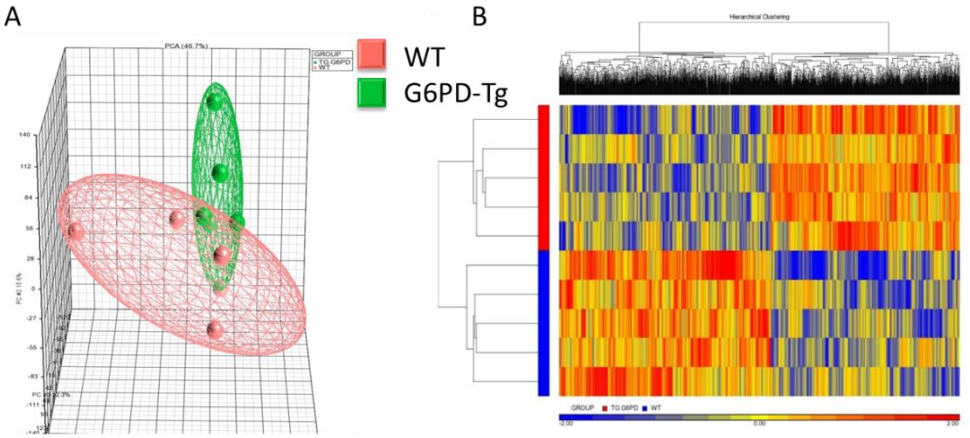

Figure 14. Transcriptomic analysis of G6PD-Tg and WT skeletal muscle samples: (A) Principal Component Analysis (PCA) showing changes in transcriptomic profile between G6PD-Tg versus WT samples. (B) Unsupervised hierarchical clustering of 1,653 differentially expressed genes, (n=4 WT; n=4 Tg).

To evaluate in what extent the overexpression of G6PD in transgenic mice affect the expression of other genes, we performed a global gene expression analysis from skeletal muscle samples of G6PD-Tg and WT old mice. The two groups of animals were clustered into two distinct sets (Figure 15A), indicating that the gene expression profile was different, and the percentage of variability between both groups was 46,7%. The analysis highlighted 1653 differentially-expressed genes, whose 779 genes upregulated (47%), and 874 downregulated (53%) in the G6PD-Tg animals. The different pattern of gene expression

observed in the principal component analysis was confirmed by an unsupervised hierarchical clustering analysis (Figure 15B).

4.2. Differentially expressed genes involved in metabolic pathways and biological processes

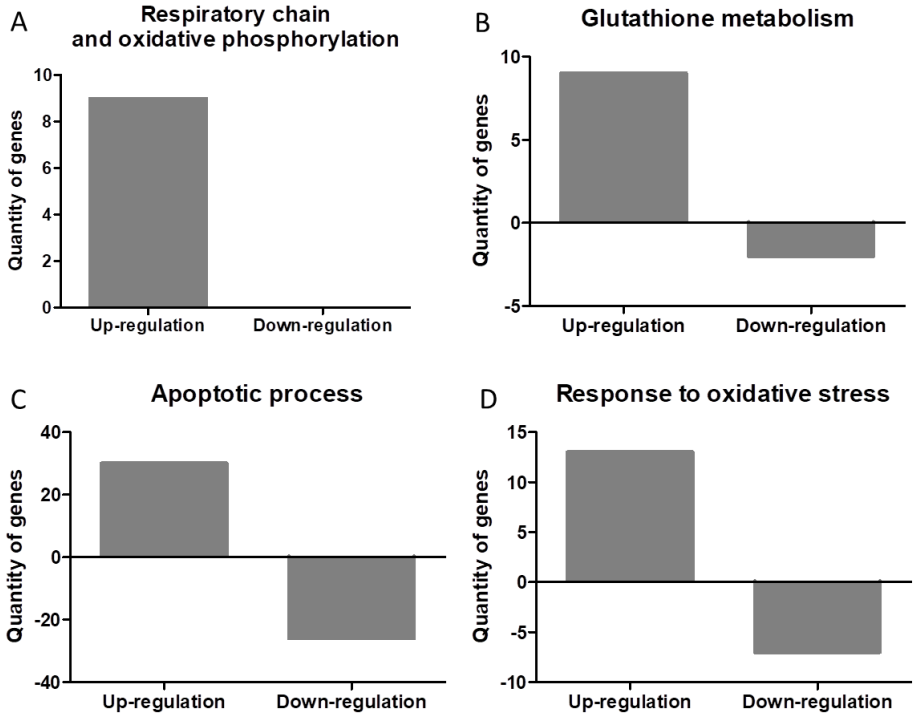

Figure 15. Quantity of genes up and down-regulated in G6PD-Tg samples versus WT: Genes involved in (A) respiratory chain and oxidative phosphorylation, (B) glutathione metabolism, (C) apoptotic process, (D) response to oxidative stress, (n=4 WT; n=4 Tg).

RNA samples from G6PD-Tg mice were separated into a different cluster from those of WT samples. In regards of our previous results obtained from muscular protein expression, we analyzed

differentially expressed genes and identified the most representative metabolic pathways. Respiratory chain/oxidative phosphorylation was one of the main affected pathway, (9 genes upregulated in G6PD-Tg mice), followed by the glutathione metabolism pathway (9 genes upregulated and 2 downregulated). Two biological processes were also highlighted, one implicating apoptosis (30 genes upregulated and 26 genes downregulated), and the other regarding responses to oxidative stress (13 genes upregulated and 7 downregulated), (Figure 8). The list of genes is presented in the following table 5.

Table 5: Genes up and down-regulated in G6PD-Tg muscles

Pathway (Number of genes)	Genes	Enrichment P-value
Respiratory chain and oxidative phosphorylation (9)	*Ndufa3, Cox7a1, Cox8a, Cox5b, Cox1, Cox6a1, Ndufb8, Ndufs8, Uqcrc2*	0.009
Glutathione metabolism (11)	*Gstm1, Gstz1, Gpx3, Gpx4, Txnrd3, Gstm2, Gstm3, Glrx, Gsta3, Anpep, Lap3*	0.035
Apoptotic process (56)	*Sh3glb1, Yars, Lalba, Gapdh, Irf3, Trp53, Epha2, Tm2d1, Satb1, Zc3h8, Trib3, C1qbp, Cited1, Prkdc, Sirt1, Psmc1, Psmc2, Psmd6, Ube2d3, Rabep1, Add1, Comp, Prkcq, Mapk3, Jak2, Bad, Ppp2r1a, Pkn2, Steap3, Fhit, Cst3, Sfrp5, Bnip3l, Psma2, Psmd12, Apip, Ece1, Mul1, Dad1, Loc102641600, Ywhaq, Srgn, Fkbp8, Tctn3, Sh3kbp1, Mtfp1, Cradd, Shisa5, Faf1, Tox3, Apopt1, Cideb, Fam3b, Rtn3, Clptm1l, Arel1*	3.53×10^{-7}
Response to oxidative stress (20)	*Gpx3, Gpx4, Ndufa6, Cox1, Ndufs8, Sod3, Duox1, Trp53, Xpa, Sirt1, Map2k1, Jak2, Mmp2, Lrrk2, Cst3, Aldh1a1, Cygb, Msra, Pdlim1, Psip1*	5.08×10^{-7}

Identify molecular signatures that may play a role in loss of muscle function with aging is a crucial issue to fight against frailty. Lee et al. (1999) already demonstrated that aging resulted in a differential gene expression pattern in skeletal muscle samples. Studies conducted in elderly population demonstrated that genes encoding proteins involved in energy metabolism, mitochondrial protein synthesis, stress responses, and apoptosis, appeared to be affected by aging (Stephen Welle et al., 2004, 2003). In animal models, inducible genes for oxidative stress, DNA damage and mitochondrial dysfunction were subjects to important transcriptional changes induced by aging in skeletal muscles (S. Park & Prolla, 2005). Zhou et al. (2015) performed a transcriptomic analysis in animals of distinct ages (young, mature, and old mice), and revealed that various cellular processes including response to oxidative stress, oxidative phosphorylation, and phosphatase activity were less active in muscles of aged animals. Besides, it has been observed in gastrocnemius muscles of old mice, a drop in the expression of a variety of transcripts encoding proteins involved in oxidative phosphorylation, mitochondrial biogenesis, oxidative stress defense, and apoptosis. These data were consistent with a previously published survey of muscle gene expression comparing young and old mice, where the expression of many genes involved in oxidative phosphorylation and

oxidative stress defense were decreased in older animals (Houtkooper et al., 2011).

Our transcriptomic analyze revealed that the moderate overexpression of G6PD enzyme impacts a large variety of genes whose implication in muscle well-functioning is crucial with aging. Because of its critical metabolic role, G6PD enzyme constitutes an interesting target in the fight against aged-induced muscle dysfunctions. The modulation of its activity and/or content would affect many physiological processes, notably through the improvement of cellular redox state.

CONCLUSION

Here, the originality of our study was to evaluate if a specific model of transgenic mice could protect against the onset of frailty. Indeed, we showed that the moderate overexpression of the G6PD enzyme in old mice appears as an effective strategy to delay frailty. The syndrome of frailty is multifactorial, but we know that skeletal muscle alterations with aging constitute the major contribution of the loss of robustness in elderly population. The underlying mechanisms conducing to the improvement of muscular function in the G6PD-Tg mice are not totally elucidated but our study highlighted some interesting clues. We demonstrated that G6PD overexpression lead to an enhancement of the glutathione antioxidant system, as well as an improved muscle quality. Moreover, skeletal muscles of G6PD-Tg mice exhibited changes in the expression of many genes involved in respiratory chain, oxidative phosphorylation and glutathione metabolism. Finally, our results indicate the G6PD enzyme as an interesting target to fight against frailty and sarcopenia. Nutritional interventions or physical exercise focused on its activation would be of particular interest in the prevention of skeletal muscle deconditioning that affect elderly people.

STUDY II

EFFECTS OF ANTIOXIDANT SUPPLEMENTATION ON HYPOACTIVITY-INDUCED MUSCLE DECONDITIONING

CONTEXT OF THE STUDY

The present study aimed to evaluate the effects of an antioxidant supplementation in the prevention of muscle deconditioning induced by long-term hypoactivity. The experiment was conducted at the Space Clinic of the Institute of Space Medicine and Physiology (Medes-IMPS, Rangueil Hospital) in Toulouse (France) and was sponsored by the European Space Agency (ESA) and the French National Space Agency (CNES). The study consisted of 60 days of hypoactivity using the well-known Head Down BedRest model (HDBR). Participants were randomly assigned to placebo or cocktail group, and muscle biopsies from the *vastus lateralis* were collected in different point of the experiment. Muscles samples allow us to evaluate muscular atrophy, myotypologic changes, and biochemical parameters of oxidative stress and protein balance pathways.

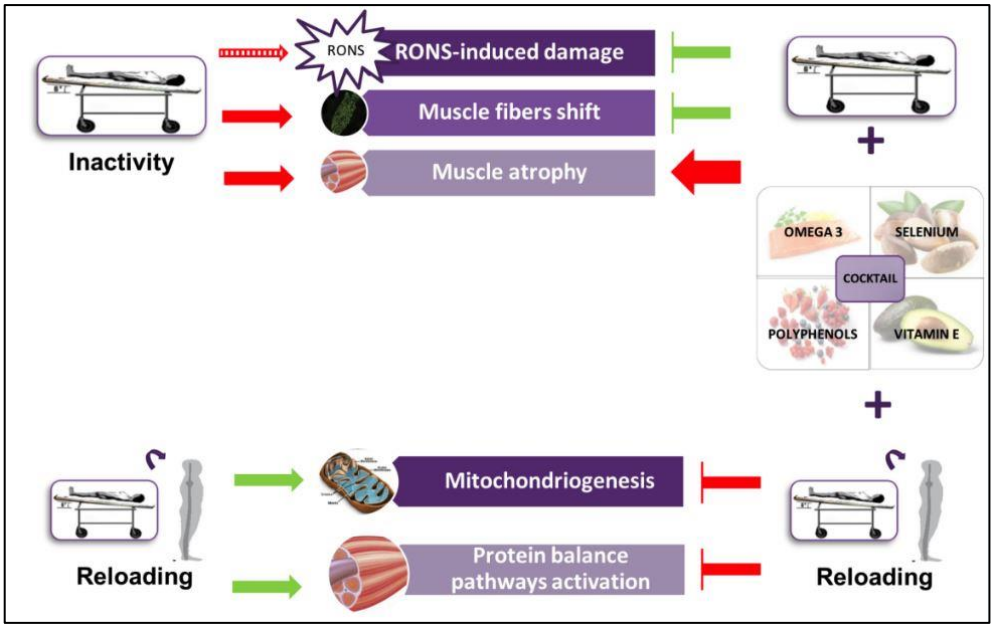

Figure 16. Graphical abstract of the study II

MATERIAL AND METHODS

1. Overall study design

1.1 Subjects and ethics statement

Twenty healthy active males were selected for this experiment. Their anthropometric data are presented in the following table 6. The subjects had no medical history or physical signs of neuromuscular disorders. They were non-smokers and were not taking any drugs or medications. All subjects gave informed consent to the experimental procedures, which were approved by the local ethics committee (CPP Sud-Ouest et Outre-Mer I, France, number ID RCB: 2016-A00401–50) in accordance with the Declaration of Helsinki. All experiments were conducted at the Space Clinic of the Institute of Space Medicine and Physiology (Medes-IMPS, Rangueil Hospital) in Toulouse (France) and were sponsored by the European Space Agency (ESA) and the French National Space Agency (CNES).

Table 6: Anthropometric data of the HDBR experiment

Subjects	Age (y)	Height (m)	BMI	Weight (pre)	Weight (post)	Weight (recovery)
A	41	1,74	22,2	68,2	66,3	68,3
B	29	1,84	22,0	76,8	71,7	73,7
C	20	1,84	23,7	83,4	80,9	83,5
D	42	1,69	22,8	62,4	61,7	63,3
E	36	1,81	25,9	86	84,4	86,7
F	44	1,75	24,6	76,9	75,7	78,1
G	25	1,81	25,4	83,5	82,6	84,9
H	24	1,72	23,3	67,7	65,7	67,1
I	39	1,68	25,0	71,7	71,2	72,9
J	41	1,76	22,7	72	69,7	72,3
K	28	1,77	24,0	76,6	77,8	79,9
L	32	1,73	24,8	77,6	74,0	75,8
M	24	1,74	22,5	70	71,1	72,9
N	27	1,71	22,5	61,6	60,3	62,3
O	35	1,77	25,0	79,3	78,2	80,8
P	44	1,8	22,2	71,1	68,3	70,9
Q	40	1,83	22,1	76,6	74,3	77,7
R	30	1,76	22,5	66,6	66,3	68,5
S	37	1,72	22,6	66,8	65,4	67,9
T	45	1,75	27,5	84,8	79,7	81,3
MEAN	**34,2**	**1,8**	**23,7**	**74,0**	**72,3**	**74,4**

1.2. Experimental protocol of HDBR

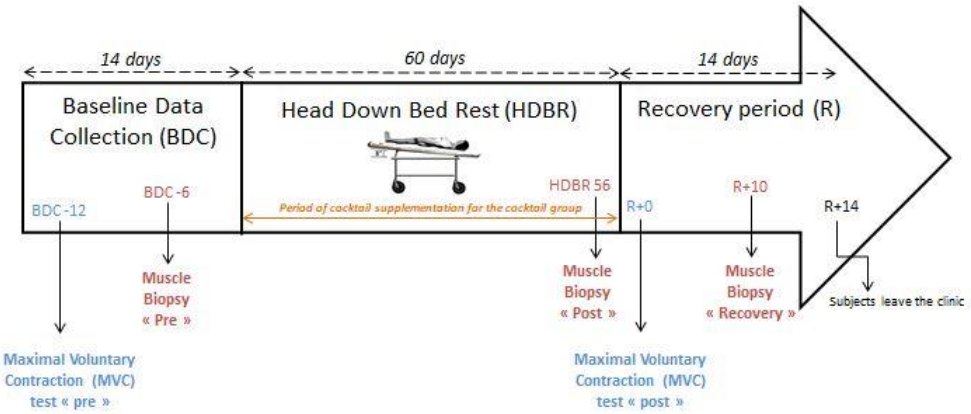

Figure 17. Time axis representative of the entire experimental design: 60 days of head down bed rest (HDBR) proceeded by 14 days of baseline data collection (BDC), and followed by 14 days of recovery.

This experiment consisted of a 2-month head-down bed rest (HDBR), with a 14-day baseline data collection period before HDBR, and a 14-day recovery period after it. During the 2-month HDBR period, the subjects were extended in a supine position with a -6° tilt to preserve simulated microgravity effects. Participants were randomly assigned to two groups on a double-blind basis. Ten of the participants were part of the "Placebo" group whereas the ten others were assigned to the "Cocktail" group, and received a daily antioxidant / anti-inflammatory cocktail during the 2-month bedrest period. The daily dose administrated was composed of 530mg of polyphenols, 138mg of vitamin E, 80µg of selenium and 2,1g of omega-3. Pills were taken at

mealtimes to reduce the risks of secondary effects affecting gastrointestinal area. Each subject had a daily medical examination and the MEDES team took several standardized measurements. Room lighting was on between 07.00 and 23.00h. During the whole hospitalization phase, diet was monitored, the meals were defined by the MEDES nutritionist and provided by Toulouse Hospital.

2. Measurements of maximal isometric voluntary contraction (MVC)

To characterize the strength of the subjects' lower limbs muscles, the maximum voluntary isometric contraction (MVC) strength was measured using a ConTrex device. The left leg of the participants was used to determine strength in knee and ankle flexors and extensors muscle groups. MVC was determined at an angulation of 80° for the knee and 0° for the ankle compared to the standard referential. Two measurements were completed, the first one was performed 11 days before the bedrest protocol and the second one just before the end of bedrest (day of restart of the upright posture). To carry out these tests, each participant was familiarized with the equipment and the standardized protocol before conducting the tests. During the assessments, participants were seated on the chair of the ConTrex device and firmly attached to it to avoid disturbance of movement. The protocol was similar for each muscle group: after a short warm-

up in neutral position, series of measurements were recording with a 30-second recovery interval. Each series consisted of a first extension movement followed by an isometric contraction, and a flexion movement followed by an isometric contraction. Each one was maintained between 5 and 7 seconds and two minutes of recovery were provided every 3 sets of measurements. The total duration of the test was 15 minutes per pair of agonist/antagonist muscle group. To determine the MVC, the maximum strength level (N.m) achieved during the test was retained.

3. Muscle biopsies

Skeletal muscle biopsies were performed before (*pre*), at the end (*post*), and 10 days after the end of the HDBR period (*recovery*), from the *vastus lateralis* muscle according to a well-established method using a 5 mm Bergström biopsy needle under sterile conditions and local anesthesia (1% lidocaine). The three biopsies were obtained from the same leg of each subject (the right leg), as near to each other as possible because of potential anatomical variations. For each biopsy, one piece was immediately embedded in small silicone casts filled with a cryoprotectant (OCT, Sakura Finetek), immediately frozen in liquid nitrogen cooled isopentane, and stored at -80°C until further

histological analysis. The other piece was rapidly frozen in liquid nitrogen and stored at -80°C for biochemical analysis.

4. Analysis of muscle samples

4.1. Cryosectionning and immunohistochemistry

To determine cross-sectional area (CSA) and muscle fiber typing, transverse serial cross sections (10 μm thick) of vastus lateralis samples were obtained using a cryostat maintained at -25°C. Before labelling, the sections were dried and fixed for 10 min in acetone. The sections were then washed in phosphate-buffered saline (PBS), blocked and permeabilized for 1h with 1% Triton - 10% horse serum. The sections were incubated with anti-MyHC primary antibodies (types I; II and IIa) for 1 h at 37°C, followed by washes in PBS and incubation with the secondary antibody for 1 h at 37°C. Type IIx fibers were not stained but were deducted thanks to type II and IIa labelling. Prior to being mounted with fluorescent mounting media, slides were co-stained with DAPI, whose labelling allow us posteriors settings adjustments. After images acquisition thanks to an epifluorescence microscope (AxioScan Zeiss), fiber typing and fiber size were semi-automatically determined and analyzed using ImageJ (1.45s version) software.

4.2. Western-blotting

Muscle samples (about 50mg) were homogenized in 10 volumes of lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 100 mM NaF, 5 mM Na_3VO_4, 1% Triton X100, 1% sodium dodecyl sulfate (SDS), 40 mM β-glycerophosphate, and protease inhibitor mixture (P8340; Sigma-Aldrich)] and centrifuged at 10 000 g for 10 min (4°C). The protein content of the supernatant was determined by the bicinchonininc acid (BCA) method (P. K. Smith et al., 1985).

Sixty micrograms of protein extract were loaded into Stain-Free 4–20% precast gels (4568095; Bio-Rad) before electrophoretic transfer onto nitrocellulose membranes (Bio-Rad; Trans-Blot Turbo Blotting System). After transfer, the membranes were blocked with 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20 (Tris-buffered saline-T) containing 5% skimmed milk or BSA and incubated overnight at 4°C with primary antibodies. The membranes were then incubated for 1 h with a peroxidase-conjugated secondary antibody. The immunoblots were revealed using a Pierce ECL kit (32106; Thermo Scientific), and proteins were visualized by enhanced chemiluminescence using the ChemiDoc Touch Imaging System and quantified with Image Lab™ Touch Software (version 5.2.1). Stain-

Free technology was used as the loading control. A large number of methodological studies have already validated this technology and explained its functioning in detail (Colella et al., 2012; Vigelsø et al., 2015)

4.3. Antibodies

Primary antibodies	Reference	Commercial	Dilution
p-4EBP1	9451	Cell signaling	1:1000
4EBP1	9644	Cell signaling	1:1000
4HNE	46545	Abcam	1:1000
ATF4	11815	Cell signaling	1:500
ATG7	8558	Cell signaling	1:1000
Atrogin	Sc-33782	Santa Cruz	1:200
Bax	Sc-493	Santa Cruz	1:200
Bcl2	Sc-7382	Santa Cruz	1:200
Catalase	110704	Genetex	1:1000
Citrate synthase	Sc-390693	Santa Cruz	1:200
COX IV	Sc-69360	Santa Cruz	1:200
Cytochrome c	Sc-13560	Santa Cruz	1:200
p-Eif2α	3398	Cell signaling	1:1000
Eif2α	9722	Cell signaling	1:1000

Fis1	Sc-98900	Santa Cruz	1:200
G6PD	8866	Cell signaling	1:1000
Gpx	3206	Cell signaling	1:1000
LC3	L7543	Sigma	1:400
Mfn2	9482	Cell signaling	1:1000
Murf1	Sc-27642	Santa Cruz	1:1000
p-p38	9211	Cell signaling	1:1000
p38	9212	Cell signaling	1:1000
p-p70	9206	Cell signaling	1:1000
p70	2708	Cell signaling	1:1000
Parkin	Sc-32282	Santa Cruz	1:200
p-PRAS40	13175	Cell signaling	1:1000
PRAS40	2691	Cell signaling	1:1000
p-RPS6	5354	Cell signaling	1:750
RPS6	3944	Cell signaling	1:750
PINK	23707	Abcam	1:1000
PDGFRα	Sc-431	Santa Cruz	1:200
PGC1α	AB3242	Millipore	1:1000
FABP4	3544	Cell signaling	1:500
SOD1	100554	Genetex	1:1000

SOD2	116093	Genetex	1:1000
Ubiquitine	Sc-8017	Santa Cruz	1:2500
p-ULK1	4776	Cell signaling	1:1000
ULK1	6888	Cell signaling	1:1000
MHC	05-716	Millipore	1:3000
TNFα	Sc-52746	Santa Cruz	1:1000
Immunohistochemistry			
Anti-MyHC1	BA-D5	DSHB	1:10
Anti-MyHC2	My-32	Sigma-Aldrich	1:200
Anti-MyHC2a	SC-71	DSHB	1:10
Anti-laminine	L9393	Sigma	1:200
Secondary antibodies			
Anti-mouse	7076	Cell signaling	1:5000
Anti-rabbit	7074	Cell signaling	1:5000
Anti-goat	Sc-2953	Santa Cruz	1:4000
Alexa 488	A11034	Invitrogen	1:800
Alexa 588	A11031	Invitrogen	1:800

5. Statistical analysis

All values are expressed as the mean ± SEM, and the significance level was set at $p< 0.05$. Normal distribution of the samples was assessed by the Shapiro-Wilk test. To compare our two different groups and their different conditions (pre-post-recovery), we used a two-way repeated measures ANOVA. In case of non-normal data, we used a Friedman ANOVA. Statistical analyses were made using Statistica and GraphPad Prism softwares, and all graphs were realized with GraphPad Prism5 Software.

RESULTS AND DISCUSSION

1. Evaluation of muscle strength and muscle mass

1.1. Loss of muscle strength

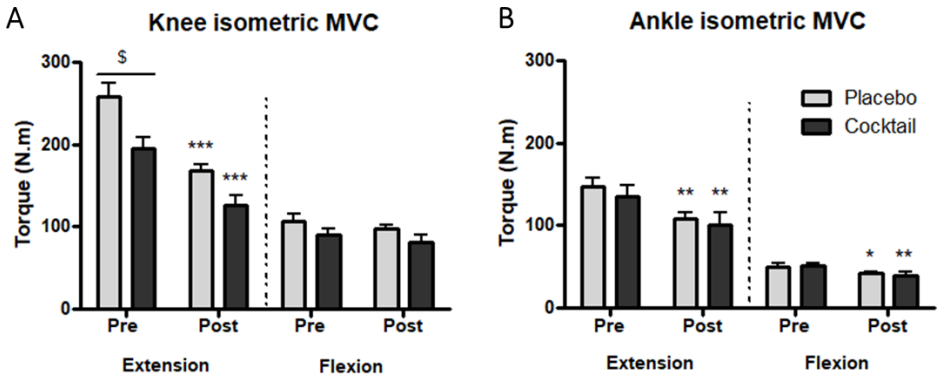

Figure 18. Isometric maximum voluntary contractions (MVC) torques before and after the HDBR experiment: Torques (N.m) generated during isometric MVC in knee (A) and ankle (B) extension and flexion positions. Data bars represent means ± SD. *p<0.05, **p<0.01, ***p<0.001: difference between time condition (pre vs post). $p<0.05: difference between groups (cocktail vs placebo).

To evaluate the changes in skeletal muscle strength due to HDBR, we measured isometric maximum voluntary contractions (MVC) represented as torques (N.m), before (pre) and after (post) the two months of bedrest. Measurements were recorded for knee and ankle articulations, in extension and flexion positions. Torques measured in knee extension were significantly decreased after bedrest in placebo (-35%), as well as in the cocktail group (-36%), whereas any differences

were observed for the flexion modality (Figure 19A). In ankle extension and flexion, torques were lower after bedrest in the placebo subjects as well as the supplemented ones. Diminutions reached -25% for both groups in extension modality, and -16% (placebo) and -22% (cocktail) for the flexion position (figure 19B). These results demonstrated that 2 months of bedrest induced a significant loss of lower limbs muscle strength, which one was not prevented by cocktail supplementation.

1.2. Atrophy of muscle fibers

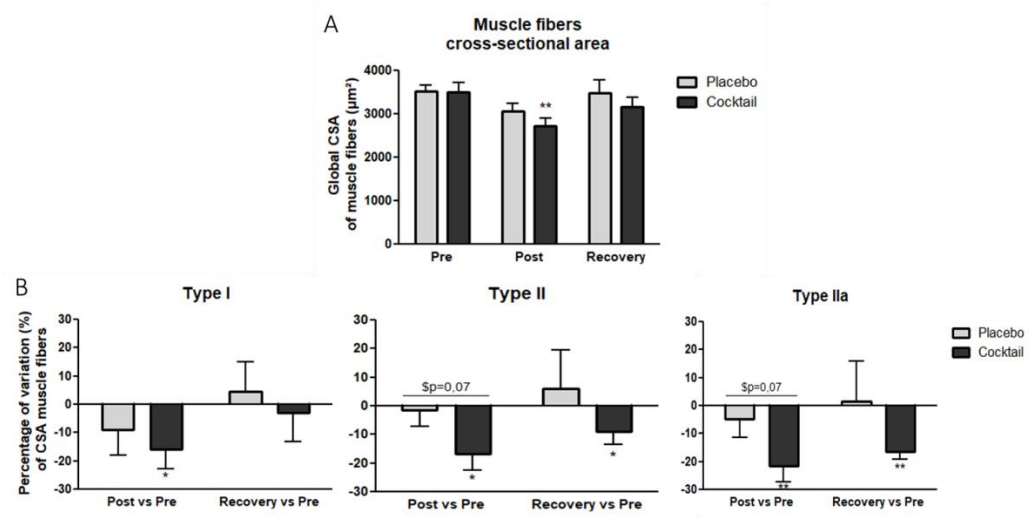

Figure 19. Cross-sectional areas (CSA) of muscle fibers from vastus lateralis biopsies: (A) Global CSA of muscle fibers (µm²) before (pre), after (post), and 10 days after the end (recovery) of 2 months of HDBR. (B) Variation of CSA of Type I, Type II and Type IIa muscle fibers, between different time conditions. Data bars represent means ± SEM. *p<0.05,**p<0.01: difference between time condition. $p<0.05: difference between group treatment (cocktail vs placebo).

Cross-sectional areas (CSA) of global, and specific fiber types, were identified from biopsies of *pre*, *post*, and *recovery* conditions (Figure 20A). Cocktail group demonstrated a -22,5% of decrease in muscle fiber CSA after bedrest (from 3511 μm^2 before, to 2720μm^2 after bedrest). The same tendency is observed in placebo group (-11,7%) although statistical analysis does not permit to see difference. After 10 days of reloading at the end of the bedrest, placebo subjects recovered values of CSA similar to those observed before bedrest (3525μm^2 in *pre* vs 3485μm^2 in *recovery*). This was not the case for the supplemented subjects, who did not recover basal values (3511μm^2 in *pre* vs 3169μm^2 in *recovery*). We next evaluated the variations of CSA of each specific fiber types, between *pre* and *post* conditions, and comparing *recovery* point to the basal one (Figure 20B). We observed that every types of muscle fiber were atrophied in the cocktail group after 2 months of bedrest (-16% type I; -17% type II and -21% type IIa). The fast fiber types (II and IIa) were more affected and do not recovered their original CSA after 10 days of reloading (*recovery* point).

These results highlighted that cocktail was not able to prevent skeletal muscle atrophy. Indeed, cocktail subjects were even more affected by

myofiber atrophy, and particularly their glycolytic type II muscle fibers.

1.3. Changes in myofibers type distribution

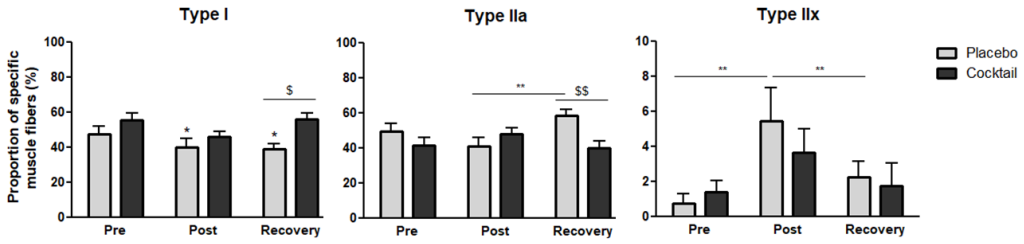

Figure 20. Distribution (%) of muscle fibers in the different time-points and conditions of the HDBR experiment: Proportion of Type I, Type IIa and Type IIx muscle fibers before (pre), after (post), and 10 days after the end (recovery) of 2 months of HDBR. Data bars represent means ± SEM. *p<0.05, **p<0.01: difference between time condition. $p<0.05, $$p<0.01: difference between group treatment (cocktail vs placebo).

In placebo group, two months of bedrest induced a decrease in the percentage of fiber expressing MyHC I (slow type). In this group, the proportion of fibers expressing MyHC IIa was higher after 10 days of recovery than at the end of the bedrest (*post*). In cocktail group, we observed, at the recovery point, a higher proportion of type I fibers than placebo group, as well as a lower proportion of type IIa fibers. Focusing on fast fibers expressing MyHC IIx, we observed in placebo group a dramatic increase of their proportion between pre and post condition, but tend to return to basal values after a short recovery. However, this is not observed in cocktail group, which suggest a

possible protective effect of the cocktail in the expression of IIx fibers (Figure 21). These findings regarding myotypologic changes point that a prolonged inactivity modulates muscle composition, reducing the part of slow fibers (type I) for the benefit of fibers expressing MyHC IIx, and that the cocktail is able to prevent this classical parameter observed in unloading and microgravity situations.

1. Discussion

After 2 months of HDBR, all subjects presented muscle deconditioning characterized at the functional level by a loss of muscle strength. Isometric MVC of quadriceps and triceps sural muscle groups were decreased in placebo (-32% and -21% respectively) and cocktail subjects (-33% and -26% respectively). Previous studies using the same protocol duration (60 days of HDBR) already founded a loss of lower limbs muscle strength (Kramer et al., 2017; T. A. Trappe, Burd, Louis, Lee, & Trappe, 2007). For a longer HDBR period, (90 days), Alkner & Tesch (2004) observed a decrease of 51% of MVC in knee extension and -45% in ankle extension. Loss of muscle strength was also observed in shorter hypoactivity experiments. For example, Zachwieja et al. (1999) founded a decrease of quadriceps muscle strength (-17%) after 28 days of bedrest. Recently, using the dry immersion model of simulated microgravity,

our research group showed that few days of hypoactivity induced a decrease of 9% of MVC (Demangel et al., 2017). Concerning this first functional parameter, our results show no effects of cocktail supplementation on muscle strength loss.

It is known that inactivity-induced loss of force is the consequence of a variety of factors: atrophy of muscle cells (Chopard, Hillock, et al., 2009), innervation deficiency (Kawakami et al., 2001), dysregulation of intracellular calcium machinery (Thom et al., 2001), and fatty infiltrations (Delmonico et al., 2009; Pagano et al., 2018). Among these elements, amyotrophy plays a predominant role. In our study, cocktail supplementation did not prevent muscle atrophy, and even accentuated the decrease in muscle fiber CSA after the 60 days of HDBR compared to the placebo group. Global CSA (-22%), as well as type I, type II (-16% both) and type IIa, (-21%) muscle fibers of supplemented subjects exhibited significant atrophy after bedrest. More particularly in this group, fast muscle fibers (type II) were more affected than the slow ones (type I). In humans, literature already described that fast fibers may be more susceptible to microgravity-induced atrophy than oxidative fibers (Edgerton et al., 1995; Fitts et al., 2000; Widrick et al., 1999). Muscle deconditioning is also characterized by changes in muscle fiber typology. In the present study, all subjects exhibited the same fiber type distribution before the

experiment. However, 10 days after the end of HDBR (*recovery*), cocktail subjects presented a higher part of type I fibers to the detriment of type IIa (56% of type I and 40% of type IIa), comparing to placebo subjects (39% of type I and 58% of type IIa). In placebo group, the increase of IIx fibers proportion (+718%) after bedrest traduced contractile properties modification induced by hypoactivity. Interestingly, cocktail supplementation seems to limit the expression of IIx fibers classically observed during muscle deconditioning. Various animals' studies focusing on microgravity-induced atrophy demonstrated a decrease of the part of slow fibers concomitant with an increase in type IIx MyHC expression (D. L. Allen et al., 1996; Caiozzo et al., 1996; F Haddad et al., 1993). The same tendency was also described in human experiments. For example, Edgerton et al. (1995) reported a diminution from 48% to 40% of type I fibers after 11 days of spaceflight. In our study, placebo subjects exhibited the same rate of reduction (47,8% in *pre* vs 40% in *post* condition). We can establish a comparison with the results of Desaphy et al. (2010), who evaluated the effects of an antioxidant supplementation during a hindlimb suspension period in mice. The administration of trolox, a vitamin E analog, was unable to protect disused muscles from atrophy but partially prevented the MyHC isoform redistribution in soleus muscles of the supplemented animals. Indeed, trolox treatment

prevented the apparition of type IIb fibers and limited the increase of type IIx fibers induced by unloading condition. In our study, the cocktail supplementation seems to have a similar beneficial effect against the glycolytic shift of muscle composition, but there are no other studies available to discuss the mechanisms by which antioxidants interfere with the phenotypic shifts induced by hypoactivity.

Regarding the loss of muscle mass, our findings are contrary to the initial hypothesis, *i.e* a potential supplementation's protection against muscle wasting. Interestingly, although atrophy levels were higher in cocktail group, the magnitude of strength loss was equivalent in both groups. It may indicate that antioxidant supplementation would have reduced the relative part of other factors responsible for strength diminution. A possible explanation could be a better excitation-contraction coupling due to less oxidation of the ryanodine receptor (RyR). These channels, especially present as RyR1 isoform is skeletal muscles, are responsible for the release of Ca^{2+} from the reticulum sarcoplasmic necessary to the initiation of muscle contraction. It is known that RyR1 are sensible to redox state and are finely regulated by oxidation and nitrosylation processes (Mei et al., 2013; Santulli, Nakashima, Yuan, & Marks, 2017; Zissimopoulos & Lai, 2006). These modifications can lead to Ca^{2+} leak and contribute to muscle weakness

in some pathologies (Bellinger et al., 2009). For example, Andersson et al. (2011) showed that RyR oxidation in old mice triggers intracellular Ca^{2+} leak and muscle weakness. Respect to our study, the same phenomenon may have occurred in disused muscles with hypoactivity; the antioxidant cocktail may have cause less oxidation of the RyR in the supplemented subjects, explaining why the loss of strength was not higher for those subjects despite of a greater atrophy. Extensive investigations are necessary to evaluate the part of every different factor in muscle strength loss contribution.

2. Oxidative stress parameters

2.1. Markers of RONS-induced damage

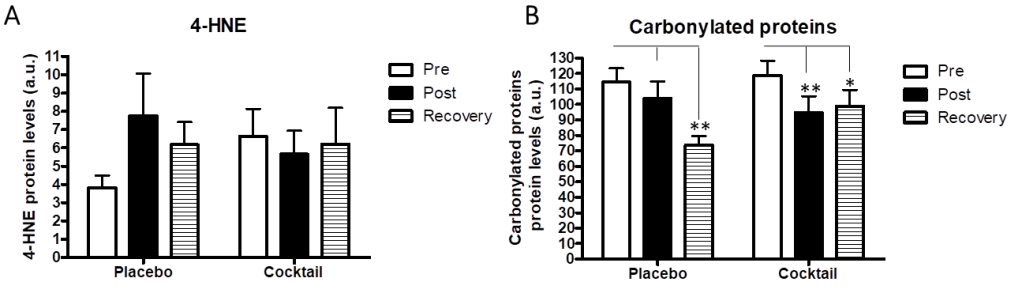

Figure 21. ROS-induced damage markers in the different time-points and conditions of the HDBR experiment: Determination of oxidative damage to lipids using 4-Hydroxynoenal (A) and carbonylated protein levels (B). Data bars represent means ± SEM. *p<0,05,**p<0,01: difference between time condition.

To evaluate ROS-induced damage to macromolecules, we analyzed the levels of 4-hydroxynonenal (4-HNE), a marker of lipid peroxidation, and those of carbonylated proteins in our muscle samples. 4-HNE levels tend to be increased in placebo subjects after bedrest, but not in the cocktail group (figure 22A). We observed that cocktail induced a decrease in the levels of oxidized proteins during bedrest. These levels were significantly decreased in both groups after 10 days of recovery (figure 22B).

2.2. Antioxidant enzymes

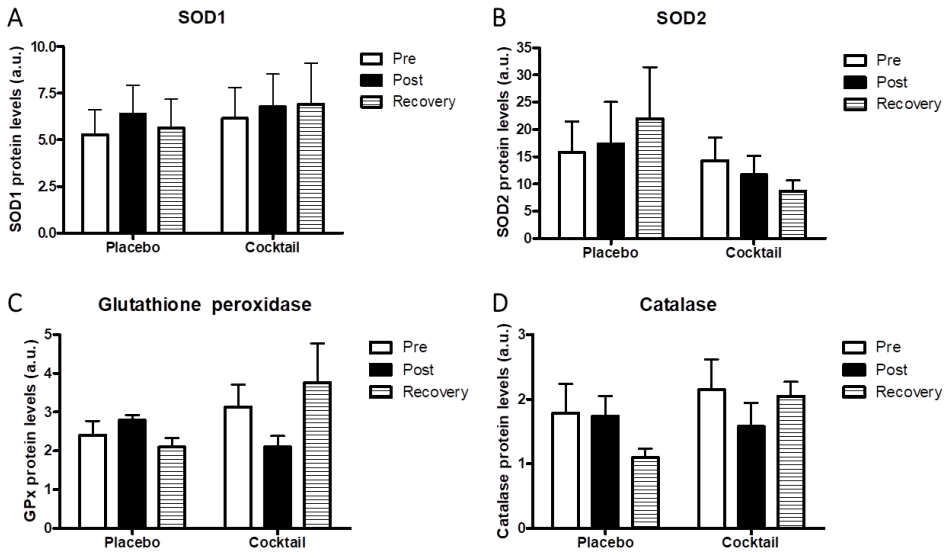

Figure 22. Antioxidant enzymes in the different time-points and conditions of the HDBR experiment: Protein content of Superoxide dismutase 1 (A) and 2 (B), Glutathione peroxydase (C) and Catalase (D). Data bars represent means ± SEM.

To explain oxidative damage, we looked at the expression of endogen antioxidant defense and measured the levels of SOD1, SOD2, GPx and catalase protein levels in muscles. No significant differences between groups or time conditions were founded (figure 23). However, we observed the same trend for the two enzymes GPx and catalase, in recovery point: placebo group seemed to have lower contents compared to the cocktail group. Our results underlined a potential protector effect of cocktail supplementation regarding the oxidative damage in muscle, although any changes in antioxidant enzymes were founded after 2 months of hypoactivity.

2. Discussion

Situations of reduced activity are known to be source of cellular RONS production (Margaritis, Rousseau, Marini, & Chopard, 2009; Powers et al., 2011). In skeletal muscles, RONS induced damage to macromolecules such as lipids, proteins and DNA. Kondo et al. (1991) was the first study hypothesizing the contribution of redox disturbances to muscle atrophy and reported that prolonged inactivity was associated with high levels of lipid peroxidation in rats. Here, after 60 days of HDBR, 4-HNE marker representing lipid peroxidation tended to increase in placebo group, whereas lower levels of carbonylated proteins were observed in supplemented subjects. These

results proved that the antioxidant supplementation appears to protect against RONS-induced damages. Nevertheless, these positive effects on macromolecules did not transcribe a better protection against muscle atrophy. To explain it, we hypothesize that cocktail could be too rich in antioxidant, and would have abolished benefic role of RONS. Indeed, minimal amounts of these molecules are necessary to the well-functioning of various physiological processes. If RONS are present in huge quantity, oxidative stress occurs, whereas absence of RONS induces "reductive stress" (Narasimhan & Rajasekaran, 2015).

In the literature, very few studies already measured RONS-induced damage in muscle biopsies, and studies measuring oxidative stress parameters after HDBR protocols described quite varied results. Libera et al. (2009) demonstrated a two fold increase of muscle carbonylated proteins levels after 5 weeks of bedrest, which was correlated with the decrease of fiber CSA. In a study using the same bedrest duration as ours (60 days), it has been observed an increase in serum samples of two major oxidative stress markers, MDA and 8-OHdG (Rai et al., 2011). However, Zwart et al. (2009) looked at various markers of oxidative stress (lipid peroxides, 8-OHdG, total antioxidant capacity, glutathione reductase, glutathione peroxidase and SOD) before and after 21 days of bedrest and do not found any

variation. After 1-month-long HDBR, the same authors observed an increase of SOD, as well as a decrease of the total antioxidant capacity, the first week of recovery following 3 months of HDBR. (Sara R Zwart et al., 2009). The paper of Stein et al. (1999) described the evolution of oxidative damage to membrane lipids and DNA after long-term (4 to 9 months) and short-term (17 days) spaceflight, as well as after 17 days of simulated microgravity. They observed an increase of 8-OHdG levels after long-term, but no changes after short-term spaceflight. After the 17 days of bedrest, no changes were founded, but 8-iso-prostaglandin F2α (8-iso-PGF2α) levels were increased during the recovery phase. Besides, antioxidant enzyme activities were analyzed in erythrocytes of healthy men subjected to 60 and 90 days of HDBR. The activity of glutathione peroxidase was increased after 90 days but not after 60 days, whereas no changes were detected for SOD and glutathione reductase. Moreover, TBARS and plasma total thiol group concentrations (lipoperoxydative markers) stayed unchanged after both conditions (Margaritis et al., 2009). Finally, in animal model, Min et al. (2011) showed a positive effect of the antioxidant molecule SS-31 against oxidative damage in muscles. After 14 days of immobilization, the treated animals were protected against the increase of 4-HNE levels in soleus and plantaris muscles,

which constitute a result quite similar to ours, although models and protocols are obviously different.

3. Mitochondrial parameters

3.1. Oxidative metabolism markers

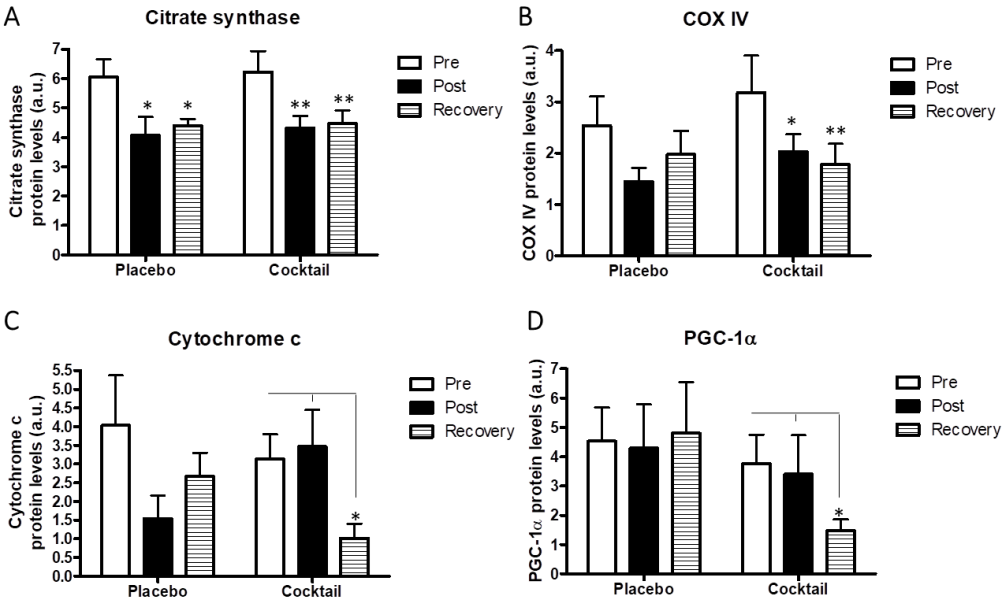

Figure 23. Oxidative metabolism parameters in the different time-points and conditions of the HDBR experiment: Determination of citrate synthase (A), COX IV (B), cytochrome c (C) and PGC1-◎ (D) protein levels. Data bars represent means ± SEM. *p<0,05,**p<0,01: difference between time condition.

To characterize changes in oxidative metabolism, we analyzed various mitochondrial parameters from the vastus lateralis samples. In the cocktail group, two important markers of mitochondrial content, Citrate synthase and COX IV, were significantly decreased after bedrest (and still 10 days later) compared to *pre* values. For placebo

subjects, this is significantly true for citrate synthase protein levels, but only a trend can be observed for COX IV levels (figure 24A, 24B). Cytochrome c, another indicator of mitochondrial content in muscles, and PGC-1α**,** the major regulator of mitochondrial biogenesis, confirmed the previous results. Lower levels of both proteins were founded in cocktail samples at the *recovery* point and attested of a rapid loss of oxidative metabolism efficacy with remobilization (figure 24C, 24D).

3.2. Mitochondrial dynamics markers

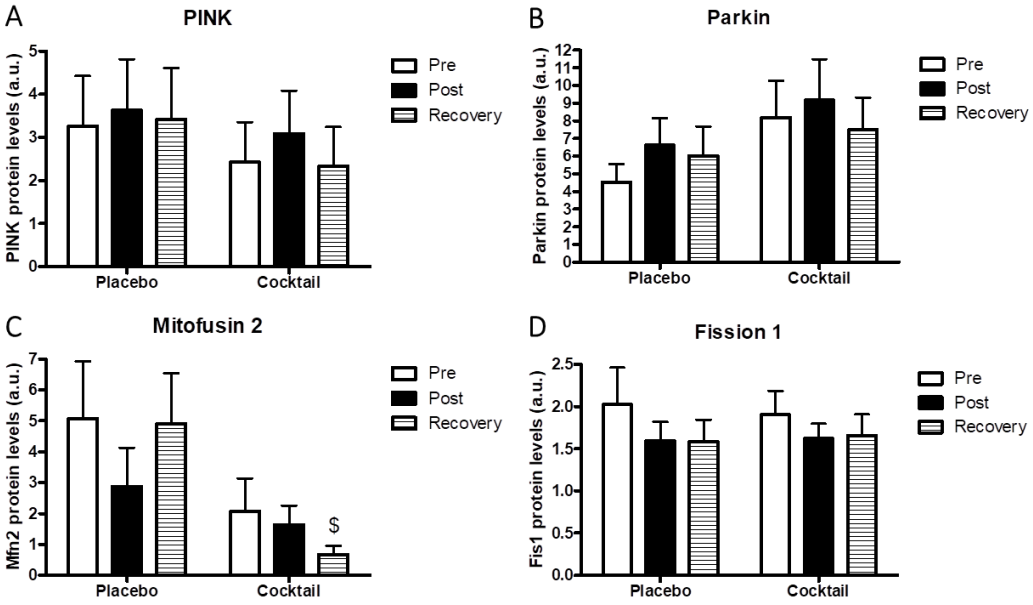

Figure 24. Mitochondrial dynamic parameters in the different time-points and conditions of the HDBR experiment: Determination of PINK (A), parkin (B), mitofusin 2 (C) and fission 1 (D) protein levels. Data bars represent means ± SEM. $p<0,05: difference between groups (cocktail vs placebo).

We also analyzed markers of mitochondrial dynamics. PINK is a mitochondrial kinase involved in mitophagy processes, and can also activates Parkin, an E3 ligase implicated in mitochondria-related ubiquitin proteasome system. Protein levels of both did not demonstrated significant variations between the bedrest conditions (figure 25A, 25B). Mitofusin2 and Fission 1 neither, the first being a regulator of mitochondrial fusion, whereas the second controls fission process (figure 25C, 25D). However, we noted that supplemented

subjects had significantly lower levels of Mfn2 in recovery point compared to the controls.

3. Discussion

Body of evidence revealed that prolonged muscle inactivity induces oxidative capacity alteration, mitochondrial dysfunction and morphologic changes, leading to atrophic pathways activation (Abadi et al., 2009; Hyatt, Deminice, Yoshihara, & Powers, 2019; Muller et al., 2007; Yajid, Mercier, Mercier, Dubouchaud, & Préfaut, 1998). The major regulator of mitochondrial biogenesis, PGC-1α, and various key mitochondrial proteins are known to be downregulated during muscle inactivity (C.-M. Chen et al., 2007; Kang, Chung, Diffee, & Ji, 2013). Brocca et al. (2010) demonstrated that hypoactivity leads to oxidative metabolism genes downregulation in animals, and these changes also occur in humans, like it has been shown in a 2-months bedrest study conducted in women (Chopard, Lecunff, et al., 2009). In a recent bedrest study, Kenny et al. (2017) observed a decrease in mitochondrial respiration in *vastus lateralis* samples, after 21 days of immobilization. Moreover, 7 days of bedrest were sufficient to reduced mitochondrial DNA, oxidative proteins expression and citrate synthase activity in human skeletal muscles (Ringholm et al., 2011). In our study, lower protein levels of citrate synthase and COX

IV after HDBR indicate a decrease in mitochondrial content in skeletal muscle of all the subjects. Moreover, 10 days of recovery were not sufficient to recover basal values. Similar results were observed in animals, after 2 weeks of immobilization followed by 5 days of recovery (Kang et al., 2013). Besides, the drop of PGC-1α and Cytochrome c levels in the *recovery* point was only observed for cocktail group. It suggests that oxidative metabolism of supplemented subjects was finally more affected than placebo subjects. Moreover, mitochondrial network is dynamic (mitochondria frequently divide and fuse) and its regulation is crucial to ensure optimal function (Dimmer & Scorrano, 2006; Youle & Karbowski, 2005). Various studies pointed an increase of fission state, concomitant with a decrease of Mfn1/2 and OPA1 levels, two major actors of fusion, as contributors of mitochondrial fragmentation during muscle inactivity (Cannavino et al., 2015; Hyatt et al., 2019; O'Leary, Vainshtein, Carter, Zhang, & Hood, 2012; Picard et al., 2015; Romanello et al., 2010). In our study, the absence of changes between the three time conditions may indicate that mitochondrial dynamics-related proteins may vary earlier, in the first days of reduced activity. After two months of immobilization, these mechanisms are certainly stabilized to ensure mitochondrial maintenance. However, in the first days following remobilization (our *recovery* point), it is interesting to note that a lower

level of Mfn2 in cocktail subjects compared to placebo is in accordance with the results for cytochrome c and PGC-1α. The sudden stop of antioxidant supplementation after 60 days certainly disturbed mitochondrial molecular pathways. More researches are needed to determine the role of antioxidant supplementation and its halt during immobilization/remobilization processes.

4. Protein balance parameters

4.1. Protein synthesis pathway

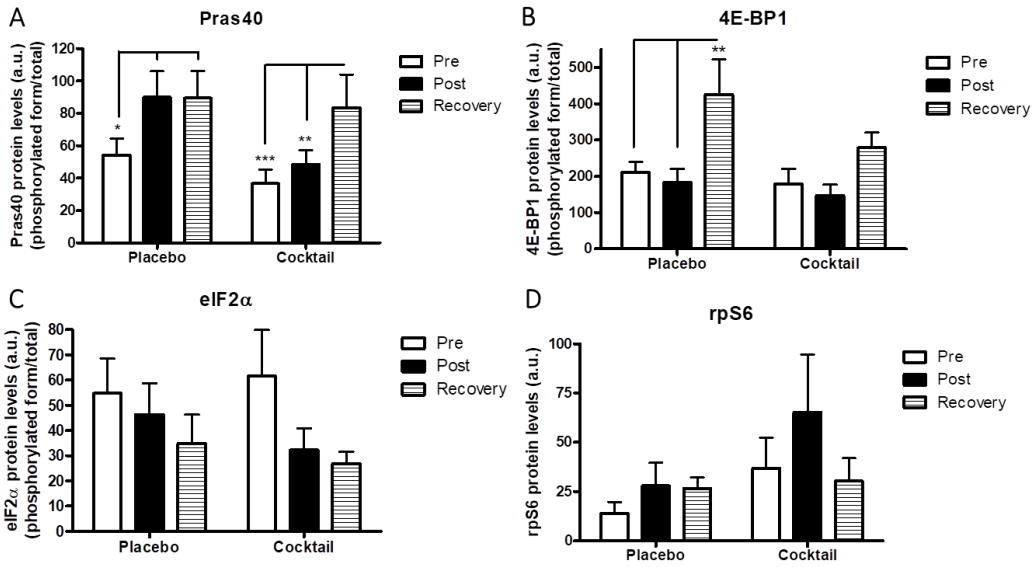

Figure 25. Markers of protein synthesis pathway in the different time-points and conditions of the HDBR experiment: Determination of Pras40 (A), 4E-BP1 (B), eIF2α (C) and rps6 (D) protein levels. Data bars represent means ± SEM. *p<0,05; **p<0,01; ***p<0,001: difference between time condition.

We evaluated changes occurred in muscle protein balance pathways in the different HDBR conditions. We first analyzed markers of the main protein synthesis pathway regulated by mTOR. Levels of phosphorylated Pras40, whose phosphorylation by Akt permits the activation of mTORC1, were increased for placebo group at the end of the bedrest (+66%, $p<0,05$; *post*) and still 10 days after it (*recovery*) respect to *pre* values (+64%, $p<0,05$). For cocktail group, no changes were observed after bedrest while a significant increase was observed at the recovery point compared to baseline (+127%, $p<0,001$) and after bed rest (+72%, $p<0,01$) (figure 26A). This result demonstrates that mTORC1 is possibly more activated after the immobilization period. The result of phosphorylated 4E-BP1, an activator of elongation processes directly phosphorylated by mTORC1, showed no difference after bedrest in both groups. However, a dramatic increase of phosphorylated 4E-BP1 was observed in the placebo group after 10 days of recovery compared to baseline and after bedrest ($p<0,01$ in both cases). Such increase was not observed in the cocktail group (figure 26B). The phosphorylation of eIF2α, which is a subunit of eIF2 initiation factor, has an inhibitory action on protein translation. Its content tended to decrease after bedrest in both groups, which means a lower inhibition of protein translation phenomenon after immobilization (figure 26C). Results regarding rps6 protein levels

show the same idea. Indeed, this target of mTORC1 is part of the ribosomal proteins family and acts so in translation processes. Although no significant variation was observed, its levels tended to increase after bedrest for both groups (figure 26D). Altogether, these results support the idea that after a long hypoactivity period, protein synthesis pathway regulated by mTOR is not downregulated, while recovery period is associated with an activation of this latter.

4.2. Protein degradation pathway

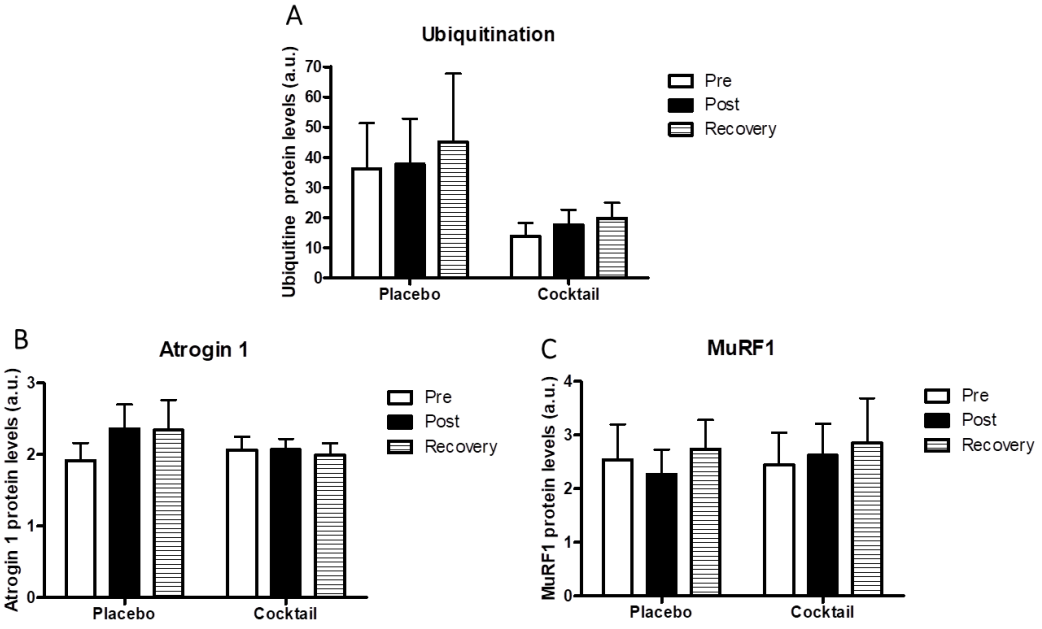

Figure 26. Markers of protein degradation pathway in the different time-points and conditions of the HDBR experiment: Determination of ubiquitination profile (A), atrogin1 (B) and MuRF1 (C) protein levels. Data bars represent means ± SEM.

On the other hand, we wanted to analyze the main markers of a major skeletal muscle breakdown pathway: the ubiquitine-proteasome system. Ubiquitination profile which detects the quantity of ubiquitinated proteins in samples did not demonstrated variations between HDBR conditions (Figure 27A). The two muscle specific E3 ubiquitin ligase, Atrogin1 and MuRF1, known to be increased under atrophy-inducing conditions were also measured, but no changes were detected (Figure 27B, 27C). It means that the major degradation pathway was not overexpressed after a long-term hypoactivity.

4.3. Autophagy pathway

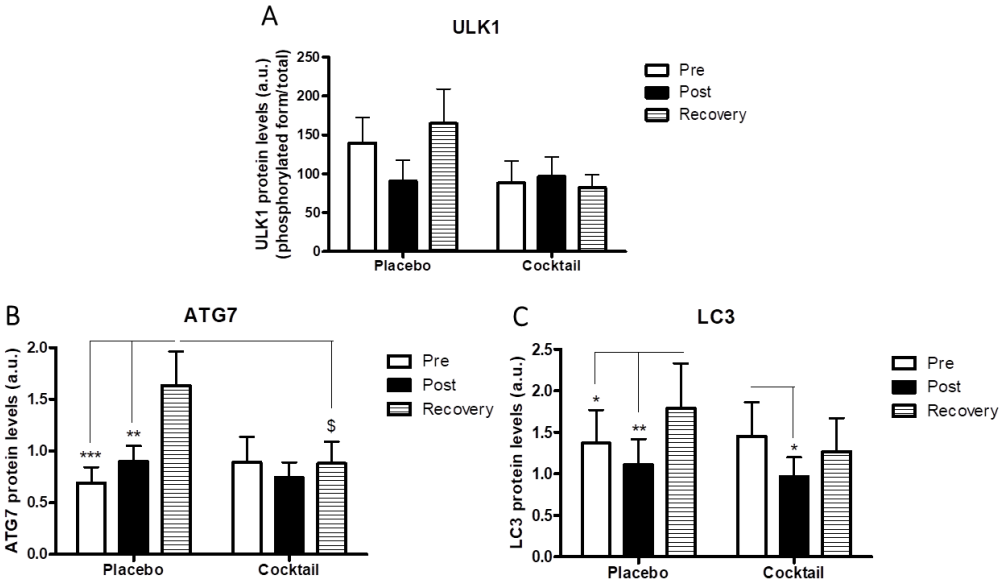

Figure 27. Markers of the autophagy lysosomal pathway in the different time-points and conditions of the HDBR experiment: Determination of ULK1 (A), ATG7 (B) and LC3 (C) protein levels. Data bars represent means ± SEM.

We also studied 3 markers of the autophagic pathway, one of the main systems responsible for skeletal muscle mass regulation. ULK1, which is inhibited by mTOR, is considered as the first initiator of the autophagic process. No significant differences were observed between conditions (figure 28A). However, the protein levels of two actors of the autophagosome formation, ATG7 and LC3 (ratio of LC3 I/LC3 II), were be higher in *recovery* point respect to *pre* and *post* conditions in placebo group (figure 28B, 28C).

4. Discussion

Research has long described that skeletal muscle hypoactivity causes a dysregulation of signaling pathways involved in muscle mass maintenance. Alterations of protein balance mechanisms occur at an early stage, from the first days of hypoactivity, and then tend to stabilize (Kawashima et al., 2004). Due to the long duration of our HDBR protocol, we especially wanted to evaluate the modulation of synthesis and degradation pathways in the days following remobilization. Analyses realized in the *recovery* point provided information regarding molecular dynamics occurring after long-term inactivity and envisage the rate of muscle recuperation. The PI3K-Akt-mTOR axis is the major pathway activating protein synthesis in skeletal muscle. Here, the elevation of Pras40 protein levels, whose phosphorylation by Akt activates the mTORC1 complex, and the increase of 4E-BP1 levels suggest an activation of the main synthesis pathway in the recovery period. This idea is strengthened by the reduction in eIF2α contents at the same time, knowing that its phosphorylation has an inhibitory action on protein translation. Data in the literature indicate that elevated RONS production can inhibit Akt/mTORC1 signaling (Powers et al., 2016). However, in our study, all subjects, supplemented or not, demonstrated the same dynamic. This indicates that the cocktail does not reduce muscle wasting

recovery processes. Our results illustrate the same insight as that described by various studies focusing on antioxidant supplementation and strength training adaptations. Indeed, this specific type of training is known to activate protein synthesis pathways and induce muscle hypertrophy. Previously, it had been described that the activation of protein synthesis actors in the overload muscles of rats was reduced by vitamin C administration (Makanae, Kawada, Sasaki, Nakazato, & Ishii, 2013). In humans, a large number of studies demonstrated negative outcomes when combining strength training protocols with vitamin C and E supplementation (Bjørnsen et al., 2016; Dutra et al., 2018; Paulsen et al., 2014). For example, after 12 weeks of resistance training, the increase in rectus femoris thickness was higher in the placebo subjects than in the supplemented subjects. All of these studies highlight that additional antioxidants may hamper the optimum activation of important hypertrophic pathways.

On the other hand, it has been previously demonstrated that oxidative stress in skeletal muscles promotes the expression of key components of the ubiquitin-proteasome system. Li et al. (2003) revealed with in vitro experiments that H_2O_2 upregulated the expression of specific E3 ligase including Atrogin1 and MuRF1. In vivo, various studies showed an increase in the expression of these two E3 ubiquitin ligase with oxidative stress (Powers et al., 2011; Talbert et al., 2013).

Unfortunately, no studies described the evolution of proteolysis markers in cases of antioxidant treatment during muscle disuse. Concerning situations of long-term hypoactivity, the paper of Salanova et al. (2008) described an overexpression of MuRF1 protein levels in atrophied *soleus* after 60 days of HDBR, but not in *vastus lateralis* muscles, which is in accordance with our proper results. Moreover, no change in the expression of MAFbx and MURF1 was reported after a 60 days HDBR study, highlighting that the up-regulation of both Atrogin1 and MuRF1 is a phenomenon occurring in early (in the first two weeks) and short-term disuse atrophy (Chopard, Lecunff, et al., 2009). We also aimed to investigate the evolution of autophagy parameters. Indeed, autophagy is a major proteolytic pathway whose activation during inactivity accentuates muscle wasting. Various reports evoked the potential of RONS to accelerate protein breakdown via this pathway (Navarro-Yepes et al., 2014; Pajares et al., 2015). The mechanisms by which RONS promote autophagy remain unclear, but they could increase ULK1 activity, the initiator of autophagy processes, through the downregulation of mTORC1. In the present study, although no significant differences in ULK1 content were observed between conditions, the levels of ATG7 and LC3II/LC3I ratio were significantly higher at *recovery* points compared with *pre-* and *post*-conditions for placebo subjects. These

results indicate an increase in key autophagy components after remobilization, but this evolution was not observed in the supplemented subjects. Moreover, the absence of a difference between *post-* and *pre-*conditions underlined that autophagy flux was not overstimulated after 2 months of inactivity. If muscle wasting is partly due to an increase of protein degradation pathways, this was not perceptible after such a long time and was certainly detectable in the first days of immobilization. Here, cocktail supplementation seems to abolish autophagy dynamics in skeletal muscle remobilization. At that day, there is still a lack of literature available regarding antioxidants and muscle autophagy pathways, but we infer that a dysregulation in favor of pro-oxidants may blunt some molecular mechanisms responsible for the control and the recovery of muscle mass. Further investigations are needed to distinguish the role of exogenous intake of antioxidants on these pathways during immobilization, and especially its impact on subsequent recovery.

5. Parameters of adipogenesis

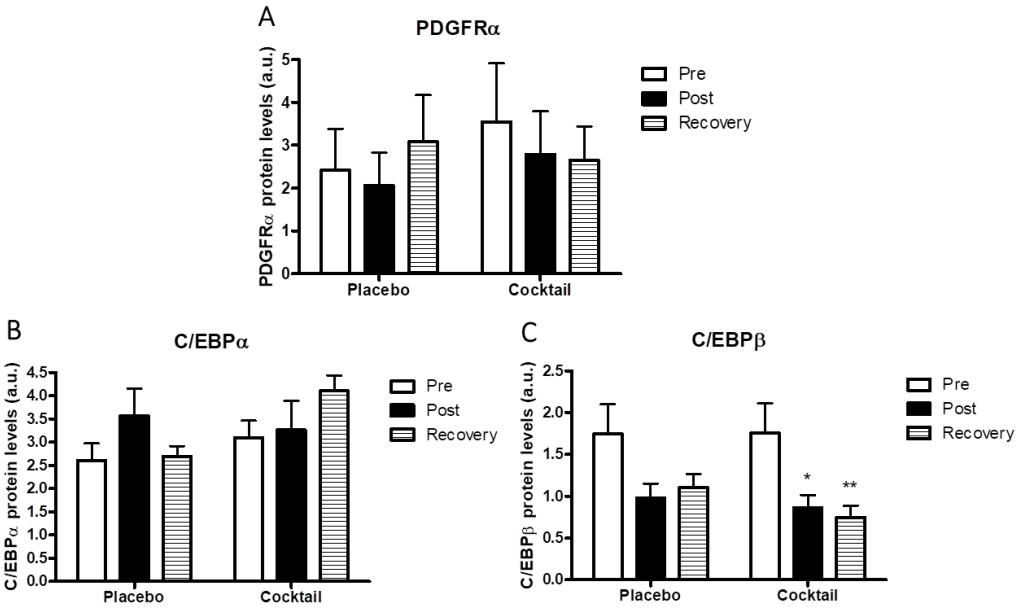

Figure 28. Markers of adipogenesis processes in the different time-points and conditions of the HDBR experiment: Determination of PDGFRα (A), C/EBPα (B) and C/EBPβ (C) protein levels. Data bars represent means ± SEM. *p<0.05, **p<0.01: difference between time condition.

We finally measured markers of adipogenesis in our muscle samples. Indeed, the accumulation of IMAT is another characteristic of muscle deconditioning. PDGFRα is a well-known marker of the FAPs (fibro-adipogenic progenitors), responsible of the formation of adipocytes in skeletal muscle. Any variation was observed in both groups for PDGFRα levels (figure 29A). Those of C/EBPα, a transcription factor acting in the latest steps of adipogenesis, did not change either (figure 29B). However, we observed a decrease in the levels of C/EBPβ, which

one is involved in the early steps of adipogenic differentiation. Its diminution in *post* and *recovery* conditions reached statistical significance in cocktail group (figure 29C).

5. Discussion

Infiltration of adipocytes in skeletal muscle tissue is a phenomenon observed in various situations linked to reduced activity (Leskinen et al., 2009; Tuttle, Sinacore, Cade, & Mueller, 2011). For example, Manini et al. (2007) observed an increase in IMAT content of 15% and 20% in thigh and calf of individuals subjected to 4 weeks of unilateral leg suspension. To date, no human study evaluated the molecular events involved in IMAT accumulation with prolonged inactivity such as our HDBR situation. In that context of simulated microgravity, only one study measured IMAT development after 3 days of dry immersion, a weightlessness model for which only 3 days are equivalent to about 10 days of HDBR situation. Besides observing an increase in IMAT content, they detected an elevation in protein levels of two transcription factors involved in the later steps of adipogenic differentiation, PPARγ and C/EBPα. The mRNA induction of this one was also increased after the 3 days of inactivity (Pagano et al., 2018). In our study, C/EBPα was not changed after bedrest certainly because adipogenic processes leading to IMAT accumulation

appeared in the very first days of a reduced activity period, and changes are not detectable after 60 days. When looking at PDGFRα, the specific marker of the FAPs in skeletal muscle, we did not observe any change in our HDBR experiment, whereas after the 3 days of dry immersion, its protein level was increased, and immunohistochemical analysis confirmed this result. These results suggest that adipose differentiation of muscle FAPs takes place immediately in the first days following reduction of activity. However, lower levels of C/EBPβ after bedrest compared to the *pre*-condition are quite surprising because it is a transcription factor of adipogenesis whose maximal levels are usually reached during the first two days of adipose differentiation (Cao, Umek, & McKnight, 1991). A possible explanation for that result can be the role of C/EBPβ as a calpains substrate. These proteolytic enzymes are involved in the degradation of myofibrillar proteins, and C/EBPβ degradation appears to be calpains-dependent (Wei et al., 2006). It makes sense regarding our myofibers atrophy findings, but measurements of skeletal muscle calpains levels would be necessary to confirm this hypothesis.

CONCLUSION

In aerospace applications, understanding muscle deconditioning mechanisms induced by microgravity environment constitutes an essential issue. The results obtained through these studies are also validated in the context of muscle deconditioning prevention, for clinical hospitalization, immobilization post injuries, or more generally chronic hypoactivity. Experimental models provide the opportunity to test countermeasures and strategies and to evaluate their effects on disuse-induced atrophy. Physical exercise is the main intervention that demonstrated positive effects (Fitts et al., 2010; Gao, Arfat, Wang, & Goswami, 2018), and some studies combined it with protein or growth factor supplementation (David L. Allen et al., 1997; Brooks et al., 2008). Despite its benefits, exercise training seems insufficient to limit muscle wasting in prolonged hypoactivity periods, and research of an efficient and feasible countermeasure including nutritional intervention is still in progress. The present study was conducted to evaluate the effects of a cocktail enriched in antioxidant/anti-inflammatory molecules in a 2-month HDBR experiment. This countermeasure was expected to limit the effects of muscle deconditioning, but our results clearly demonstrate the

ineffectiveness of this type of supplementation in the prevention of muscle mass and strength loss. Moreover, data regarding muscle molecular mechanisms highlight an alteration of recovery processes in the supplemented subjects. These results can be explained by an inhibition of the beneficial adaptations induced by the presence of RONS and illustrate the necessity of pro-oxidant molecules during long-term inactivity to maintain a certain level of muscle function. It underlines the complexity of redox balance mechanisms and demonstrates that physiological amounts of RONS are essential to activate molecular pathways and preserve positive adaptations. Indeed, a number of studies particularly in exercise training context described that over-supplementation with exogenous antioxidants impairs molecular signaling required for cellular adaptations (Chang, Huang, Tseng, Hsuuw, & Tso, 2007; M.-C. Gomez-Cabrera et al., 2008; Higashida, Kim, Higuchi, Holloszy, & Han, 2011; Merry & Ristow, 2016). In situations where subjects are strained to reduce activity, further researches are needed regarding the appropriate nutritional intervention to fight against muscle deconditioning.

GENERAL CONCLUSION AND PERSPECTIVES

These two studies demonstrated that modulate the systems of antioxidant defenses have important consequences on deconditioned skeletal muscle tissue. Indeed, muscular structure and function are disturbed in situations of aging and hypoactivity, mostly due to an imbalance of the cellular redox state. Thus, the increase of oxidative stress associated with both of these conditions justifies an intervention stimulating antioxidant defenses, in order to prevent its deleterious effects.

Our first work was dedicated to the evaluation of frailty in G6PD-Tg overexpressing mice. This genetic manipulation aimed to directly stimulate the endogenous system of glutathione, whose the efficiency is improved by elevated NADPH levels. This cofactor is directly implicated in the regeneration of GSH, which plays an essential role in cellular ROS detoxification. As the main source of NADPH, the G6PD enzyme is a pertinent target whose the over-activation has been recognized to improve the glutathione defense system. In addition to its effects on antioxidant defenses, our results demonstrated that the overexpression of G6PD was able to delay the onset of frailty in old mice. As a consequence, interventions focusing on the increase of G6PD would offer promising clues in order to prevent the apparition of sarcopenia and frailty. Therefore, our report highlighted that a stimulation of precursors of endogenous defense system could be a

good strategy to fight against muscle deconditioning in aging conditions. The study of Sinha-Hikim (2013) conduced in old mice used a quite similar strategy to prevent loss of muscle mass. Although they did not use genetic manipulation, they administrated for 6 months a cystine-based antioxidant, which is a glutathione precursor, and observed an increase of the muscular GSH/GSSG ratio characteristic of an improved RONS detoxification capacity.

In muscle deconditioning associated with elderly, data in the literature did not exhibit such positive benefits through an oral supplementation of exogenous antioxidants (Bobeuf et al., 2011; Nalbant et al., 2009). Actually, most of the studies using vitamin supplementation in older subjects did not founded the benefic expected results and some of them observed until a decrease of the expression of antioxidant genes (Cerullo, Gambassi, & Cesari, 2012; Selman et al., 2006). Consequently, research actually focuses on other strategies such as the direct activation of antioxidant enzymes. In this context, it has also been observed that the overexpression of CuZnSOD enzyme in mice, was able to prevent age-related muscle impairments (Sakellariou et al., 2014). A better understanding of the molecular pathways is needed to develop the most efficient intervention. Our findings with G6PD overexpression open new perspectives regarding the interventions that would be able to induce

the proper activation of endogenous defense systems. Thus, physical exercise or pharmaceutical molecules are potential strategies which require larger investigation.

Otherwise, various studies evidenced that vitamins supplementation showed beneficial effects in situations of muscle wasting induced by hypoactivity. It would suggest that the mechanisms regulating oxidative stress in aged muscles are distinct from those associated with inactivity in healthy subjects. In this context, the antioxidant/anti-inflammatory cocktail used in the second study of this thesis was supported by the results obtained in multiples previous studies (Beytut et al., 2018; Damiot et al., 2019; Demiryürek S, 2004; Lambert et al., 2015; Mizunoya et al., 2017; Momken et al., 2011; Servais et al., 2007). However, the unexpected ineffectiveness of the cocktail in the prevention of muscle deconditioning underlines the complexity of the processes regulating redox state. A possible explanation of these adverse effects could reside in the combination of various compounds. Indeed, experimentations combining more than 2 or 3 products are scarce. The study of Assi et al. (2015) testing a combination of polyphenols (catechins, quercetin) and vitamin C, observed an increase of skeletal muscle atrophy in cachexic mice. Moreover, combination of vitamins C, E, and β-carotene intake did not prevent loss of muscle mass and strength induced by unloading

(Koesterer et al., 2002). Here, the utilization of 4 different molecules in the cocktail is propitious to many interactions whose the consequences in terms of effects on molecular pathways remain unknown.

The second explanation for the negative effects of the cocktail supplementation would be a dysregulation of redox balance in favor of antioxidant molecules. Instead of reestablish an equilibrium, such high doses or duration of the supplementation would have abolish RONS generation or totally hamper their effects. Although RONS accumulation triggers prejudicial damage, a physiological production of RONS remains necessary for the control of various vital functions. Therefore, sudden elevation of nutritional antioxidants intake may have contribute to the installation of a "reductive stress" state (Narasimhan & Rajasekaran, 2015). This hypothesis joins the results obtained in the field of physical exercise, where antioxidants supply is worst and inhibits molecular adaptations (M.-C. Gomez-Cabrera et al., 2008; M. C. Gomez-Cabrera, Ristow, & Viña, 2012).

Moreover, it is important to underline that in our present study, the dose of the cocktail's components was the same for each individual, independently of their specific needs. In this respect, the group of Paschalis et al. published two interesting studies which highlight the

importance of adapting antioxidant supplementation to the personalized requirements of the subjects. In both studies, physical performance and oxidative stress protection were improved only in subjects who exhibited low initial concentrations of vitamin C or, glutathione, respectively. These results showed that the effectiveness of antioxidant supplementation largely depends on the redox status of the subjects receiving the treatment (Paschalis et al., 2016; Paschalis, Theodorou, Margaritelis, Kyparos, & Nikolaidis, 2018).

Finally, the conclusions of our two studies gave clues on the suitable antioxidant modulation strategy for the prevention of skeletal muscle deconditioning. It seems preferable to focus on the stimulation of endogenous defense system whether than towards exogenous supply of nutritional antioxidants. Nevertheless, the complexity of redox signaling requires better understanding to optimize countermeasures in muscle wasting situations.

RESUMEN EN ESPAÑOL

Introducción: El sistema muscular tiene un papel fundamental en el funcionamiento del organismo. Él es responsable de funciones como la producción de movimientos, la locomoción, el mantenimiento postural y el equilibrio, todas ellas imprescindibles para las actividades de la vida diaria. La calidad del tejido muscular es primordial en el mantenimiento de la calidad de vida y, a largo plazo, de la supervivencia. Sin embargo, la generalización del modo de vida sedentario por una parte y del envejecimiento de la población por otra, son dos fenómenos en expansión desde el siglo pasado, cuyo punto en común es la pérdida de cantidad (masa) y calidad (función) muscular. El músculo estriado representa el 40% de nuestra masa corporal y se caracteriza por su alta plasticidad, es decir su capacidad de adaptación al estrés ambiental (Harridge, 2007). El término desacondicionamiento muscular (DM) hace referencia a una desregulación del equilibrio homeostático, y es la consecuencia de un gran número de factores. Entre ellos, encontramos la inactividad, el envejecimiento, la inmovilización crónica, la micro gravedad o algunas patologías (Degens & Alway, 2006). El DM conduce a una alteración de la función muscular, y en él se pueden apreciar numerosas características. La primera de ellas es la pérdida de masa

muscular, evaluada a nivel de las fibras musculares por una disminución de su área de sección transversal (CSA). Ésta es asociada a un cambio miotipológico caracterizado por una disminución de fibras tipo I (fenotipo oxidativo), a beneficio de fibras tipo II (fenotipo glucolítico). Estas modificaciones estructurales están acompañadas de una perdida de propiedades contráctiles, en parte responsables de la disminución de fuerza y potencia muscular. Además, otros factores están implicados, como por ejemplo los factores nerviosos o la acumulación de células adiposas intermusculares (llamadas IMAT por intermuscular adipose tissue), (K M Baldwin et al., 2013; Bodine, 2013; Gillis & MacDonald, 2005). La desregulación de las vías de señalización implicadas en el mantenimiento de la masa muscular es, en gran parte, responsable de las alteraciones observables a nivel funcional. Este equilibrio está basado en el balance proteico que controlan las vías anabólicas y catabólicas con el objetivo de mantener un contenido proteico óptimo (Sandri, 2008; Schiaffino, Dyar, Ciciliot, Blaauw, & Sandri, 2013). En situación de DM, esas vías de síntesis y de degradación proteica están perturbadas, conduciendo al fenómeno de atrofia muscular.

En las últimas décadas, la investigación científica ha puesto a punto numerosos modelos experimentales (celulares, animales y humanos), que permiten el estudio de los procesos subyacentes al DM. Su uso es

esencial para la comprensión de los mecanismos moleculares implicados y la identificación de potenciales dianas, con el objetivo de probar medidas preventivas contra la perdida muscular.

El DM relacionado con el envejecimiento se conoce como sarcopenia. Esta es definida como una pérdida progresiva y generalizada de masa y fuerza muscular, asociada a un rendimiento físico bajo y una movilidad reducida (A J Cruz-Jentoft et al., 2010; Fielding et al., 2011; John E. Morley et al., 2011; Muscaritoli et al., 2010). La sarcopenia se constituye como el principal factor de aparición del síndrome de fragilidad ("frailty"), con quien comparte varias características fenotípicas comunes (Cederholm, 2015; Landi et al., 2015). La fragilidad es considerada como un decline progresivo de los sistemas fisiológicos relacionados con el envejecimiento. Es el resultado de una disminución de las capacidades intrínsecas de resistencia al estrés, aumentando los riesgos de padecer efectos negativos sobre la salud como la hospitalización, la pérdida de autonomía o la muerta precoz (Cesari et al., 2014; Clegg et al., 2013; Nascimento et al., 2019; Rodriguez-Manas & Fried, 2015). A pesar de la variedad de aspectos relacionados con el síndrome de fragilidad, se sabe que la función física y muscular tienen un papel primordial (Abellan van Kan et al., 2009; Daniels et al., 2008; Ferrucci et al., 2004). Fried et al (2001) han establecido un fenotipo de sujeto "frágil" que incluye los siguientes

criterios : pérdida de peso involuntario, agotamiento, debilidad muscular, baja velocidad de marcha y bajo nivel de actividad física. Los sujetos son considerados como "frágiles" si cumplen al menos 3 de esos 5 criterios. Esta evaluación es la más común para determinar la fragilidad en el ámbito clínico. Con el aumento de la prevalencia de este síndrome a lo largo de las últimas décadas, es primordial entender los mecanismos moleculares que contribuyen a su aparición (Puts et al., 2017). Para ello, el desarrollo de modelos de experimentación animal nos ofrece la oportunidad de estudiar dichos mecanismos y de probar estrategias destinadas a retrasar su aparición.

El balance redox se define como un equilibrio entre las moléculas pro-oxidantes y las antioxidantes, lo cual es esencial para el buen funcionamiento de las vías de señalización encargadas de regular numerosas funciones fisiológicas, como la producción de energía, la función inmunitaria o la contracción muscular (Reid et al., 1993; H. Sies et al., 1985; H Sies, 1997; Helmut Sies, 2000). Las especies reactivas de oxígeno (ERO) son el producto del metabolismo celular de los organismos vivos (B Halliwell, 1987; M. Valko et al., 2006). Según sus concentraciones en el organismo, los ERO tienen un doble papel que puede ser deletéreo o benéfico. Por ejemplo, a dosis bajas o

moderadas, los ERO son esenciales para varios procesos celulares, pero generan daños macromoleculares cuando están presentes a altas concentraciones (Barry Halliwell & Gutteridge, 2015; Marnett, 1999; Siems et al., 1995; Earl R Stadtman, 2004; Marian Valko et al., 2007; Wang et al., 1996). Para luchar contra los efectos deletéreos del exceso de ROS, el organismo cuenta con un potente sistema de defensas antioxidantes.

A final del siglo XX, Halliwell (1995) propuso una definición de antioxidante: « es una sustancia que, cuando está presente a baja concentración comparado con la de los sustratos oxidables, retrasa significativamente o previene la oxidación de dichos sustratos ». Las moléculas antioxidantes tienen como función luchar contra la formación de ERO y bloquear los ataques de radicales libres. Tienen la capacidad de "secuestrar" los metabolitos reactivos, disminuyendo así sus niveles de reactividad y facilitan también la reparación de daños inducidos por los ERO (Birben et al., 2012; Poljsak et al., 2013).

Las defensas antioxidantes cuentan con enzimas (catalasa, glutatión peroxidasa o superóxido dismutasa) y con actores no enzimáticos, entre los que se encuentran el glutatión, vitaminas C, E, carotenoides o polifenoles.

El concepto de estrés oxidativo fue descrito por primera vez por Sies (1985) como la acumulación excesiva de ROS, resultando en un desequilibrio entre su producción y su eliminación (H Sies, 1983). Una perturbación del estado redox genera daños irreversibles a proteínas, lípidos y ácidos nucleicos, que al final alteran las funciones fisiológicas y contribuyen al desarrollo de varias patologías (Bokov et al., 2004; Cross et al., 1987).

La enzima glucosa-6-fosfato deshidrogenasa (G6PD) fue caracterizada por primera vez por Warburg & Christian en 1932. Ellos descubrieron su implicación en funciones redox a nivel de los glóbulos rojos. La G6PD es una enzima expresada en todas las células del organismo, y constituye la primera etapa de la vía de las pentosas fosfatos. Permite la producción de NADPH, cuya concentración es determinante para el funcionamiento de varios sistemas antioxidantes, en particular el del glutatión. El NADPH es un cofactor de la enzima glutatión reductasa (GR), que cataliza la reducción del glutatión oxidado (GSSG) a glutatión reducido (GSH) (Mannervik, 1987; Meister, 1988; Scott et al., 1993; Helmut Sies, 1999). El GSH es el tiol no proteico más abundante en las células de mamífero y tiene un papel central en la defensa antioxidante de los organismos aeróbicos.

Teniendo en cuenta la implicación de la G6PD en la defensa antioxidante, un déficit en esta enzima constituye un factor de riesgo importante para el desarrollo de numerosas patologías (fabismo, diabetes, hipertensión, paro cardiaco…) (Bocchetta, 2003; Hecker et al., 2013; Heymann et al., 2012; Hwang et al., 2018). El déficit en G6PD es la perturbación enzimática más expandida a nivel mundial, afectando a más de 400 millones de personas (Nkhoma et al., 2009; Peters & Van Noorden, 2009). No obstante, su papel en el mantenimiento del tejido muscular ha sido poco estudiado.

El DM, y en particular los mecanismos moleculares implicados en la pérdida de masa que lo caracterizan, están influenciados por los ROS a través del control redox del recambio proteico. En este sentido, hay evidencia que indica que el estrés oxidativo contribuye a la atrofia muscular, por una parte inhibiendo la vía de la síntesis PI3K-Akt-mTORC1, y por otra parte estimulando la proteólisis mediante la activación de la autofagia y del sistema ubiquitín-proteasoma (Powers et al., 2016).

El envejecimiento ha sido extensamente estudiado y se han propuesto más de 300 teorías para explicarlo (Medvedev, 1990). Una de las teorías más aceptadas es « la teoría de los radicales libres del

envejecimiento » de Viña et al. (2007). Este teoría fue postulada por Harman en la revista "Journal of Gerontology" (1956).

Desde entonces, la teoría evolucionó y múltiples estudios, algunos a favor y otros en contra, han surgido (Viña et al., 2013).

Numerosos datos en la literatura han demostrado una desregulación del equilibrio redox durante el envejecimiento. El aumento en la producción y acumulación de daños oxidativos han sido reportados en el tejido muscular de organismos envejecidos (Drew et al., 2003; Sastre et al., 2003; Vasilaki et al., 2006). Además, una disminución significativa de las defensas antioxidantes en sujetos ancianos podría contribuir a la aparición de la sarcopenia, y en particular al síndrome de fragilidad (Baumann et al., 2016; M. J. Jackson, 2015; Kerksick & Zuhl, 2015; Scicchitano et al., 2018). Recientemente, miembros de nuestro equipo han descubierto que el estrés oxidativo está en realidad asociado a la fragilidad más que al envejecimiento en si (Inglés et al., 2014).

Así, la « teoría de los radicales libres de la fragilidad » apareció proporcionando una base científica a las intervenciones orientadas a la protección de daños oxidativos, para retrasar la fragilidad (Viña, 2019; Viña et al., 2018). Varios estudios clínicos han demostrado una correlación entre el estado frágil de los sujetos, y una elevación de

algunos marcadores de daño oxidativo (isoprostanos, 4-HNE, MDA, 8-OHdG), (Cesari et al., 2012; C. K. Liu et al., 2016; Serviddio et al., 2009; Wu et al., 2009).

Las intervenciones dirigidas al aumento de las defensas antioxidantes como estrategias contra el DM (en situación de envejecimiento o de inactividad), constituyen un campo activo de investigación (Bonetto et al., 2009; Janna R. Jackson et al., 2010; Ryan et al., 2011). En personas mayores, varios estudios demuestran una correlación positiva entre el nivel plasmático de antioxidantes (vitaminas C, E, carotenoides) y parámetros de función muscular (fuerza, velocidad de marcha) (Ble et al., 2006; A. Das et al., 2019; J.-S. Kim et al., 2010; Saito et al., 2012; Semba et al., 2003). No obstante, la mayoría de los estudios que utilizaron una suplementación nutricional con este tipo de antioxidantes, falló en demostrar un impacto positivo en la pérdida de fuerza, masa, o rendimiento muscular relacionado con la edad (Bobeuf et al., 2011; Marzani et al., 2008; Nalbant et al., 2009). Por otra parte, en la lucha contra el DM inducido por una reducción de la actividad, suplementaciones con sustancias antioxidantes (vitamina E, polifenoles, cúrcuma, β-caroteno) han demostrado efectos dispares. Sin embargo, numerosos estudios resaltan sus efectos beneficiosos contra la pérdida de masa y fuerza muscular (Beytut et al., 2018; Demiryürek S, 2004; Duarte & Soares, 1997; Kondo et al., 1991; Kondo,

Miura, Nakagaki, et al., 1992; Momken et al., 2011; Ogawa et al., 2013; Servais et al., 2007).

Objetivos: El objetivo principal de esta tesis fue evaluar el impacto de la modulación de las defensas antioxidantes sobre la prevención del DM. Este ha sido estudiado bajo dos perspectivas. La primera está vinculada con el envejecimiento, y la segunda, con la hipoactividad. Esta tesis fue conducida en dos laboratorios, lo cual permitió la elaboración de dos proyectos experimentales. El primero de ellos fue llevado a cabo en un modelo animal envejecido, y se centró en la prevención de la perdida de la función muscular relacionada con la fragilidad, mientras el segundo proyecto se enfocó en el DM inducido por hipoactividad prolongada, en humanos. Con relación a la modulación de las defensas antioxidantes, cada estudio pudo emplear dos estrategias distintas. El primer estudio utilizó una manipulación genética para estimular un actor clave del sistema antioxidante endógeno, mientras el segundo estudio se centró en el impacto de una suplementación nutricional de sustancias antioxidantes.

Estudio I: El estudio I de esta tesis es titulado: "Sobre-expresión de G6PD: efectos sobre la fragilidad en ratones envejecidos". El objetivo de este estudio fue evaluar la fragilidad en una cohorte de roedores,

usando un grupo de ratones wild type (WT) y un grupo de ratones transgénicos (G6PD-Tg) sobre-expresando la enzima G6PD.

Material y métodos: Estos ratones transgénicos fueron producidos en el Centro Nacional de Investigaciones Oncológicas (CNIO), gracias a la inserción de un fragmento genómico humano conteniendo en el gen G6PD. Desde los 18 a los 26 meses, hemos realizado una evaluación longitudinal de 5 parámetros funcionales (pérdida de peso, fuerza máxima, coordinación motora, velocidad máxima y tiempo de carrera alcanzados durante una prueba de esfuerzo en cinta de correr), y hemos calculado una puntuación estableciendo la fragilidad de los animales en cada uno de los grupos. A partir de muestras musculares de ratones de 21 meses de edad, hemos evaluado varios parámetros moleculares relacionados con el estrés oxidativo, la calidad del tejido muscular y la expresión de genes específicos. Los niveles de glutatión reducido (GSH) y oxidado (GSSG) fueron medidos por método espectrofotométrico usando el N-ethylmaleimide. La carbonilación de proteínas fue evaluada siguiendo las instrucciones del kit « oxyblot protein oxidation kit ». La peroxidación lipídica fue determinada por HPLC (cromatografía liquida de alta eficacia) mediante la determinación de los niveles de malondialdehído (MDA). La técnica del western blotting fue utilizada para medir el contenido de varias proteínas. Un análisis histológico fue realizado en cortes transversales

de músculos de ratones envejecidos para evaluar la CSA de las fibras. El análisis transcriptómico fue iniciado con una extracción de ARN total de las muestras musculares con el método del Trizol. Posteriormente, la síntesis de ADN y ARN complementarios de las muestras fue realizada siguiendo las instrucciones del kit « WT Plus Reagent Kit Manual » (Thermo Fisher), generando un chip de ADN (« microarray ») el cual contiene más de 20000 genes. Los análisis estadísticos se realizaron con los programas Statistica o GraphPad prism, con una significatividad asignada de $p<0.05$. La normalidad de los datos fue averiguada con el test de Shapiro-wilk. Para comparar dos grupos, fueron utilizados el test-t de Student o el test de Mann-Whitney en el caso de una distribución no normal. Para el "frailty score", las diferencias fueron determinadas usando en test Chi^2 de Pearson.

Resultados: Los resultados de este estudio demuestran que los ratones mayores G6PD-Tg son menos frágiles que sus congéneres WT. Hemos podido observar una tasa más elevada de GSH en los músculos de los animales que sobreexpresan G6PD, así como un ratio GSSG/GSH más bajo, indicando una mejora del sistema antioxidante del glutatión. Junto con esto, los análisis bioquímicos demostraron en los ratones G6PD-Tg una elevación de distintos marcadores indicando una mejor calidad muscular (contenido mitocondrial superior, menos

infiltraciones adiposas intermusculares, apoptosis reducida). Finalmente, el análisis transcriptómico permitió detectar una *up-regulación* de genes implicados en los mecanismos de la cadena respiratoria, la fosforilación oxidativa y del metabolismo del glutatión. Este estudio permitió plantear el primer modelo experimental que retrasa la fragilidad. En consecuencia, intervenciones nutricionales o de ejercicio físico que estimulen la enzima G6PD, serán posibles herramientas a tener en cuenta en la búsqueda de estrategias para la prevención de la pérdida de función muscular en personas mayores.

Estudio II: El estudio II de esta tesis es titulado: "Efectos de una suplementación con antioxidantes sobre el DM inducido por la hypoactividad." Este estudio tuvo por objetivo evaluar los efectos de la toma de un cocktail antioxidante sobre el DM inducido por 2 meses de encamamiento prolongado.

Material y métodos: La experimentación fue realizada en la clínica espacial de Toulouse (Medes-IMPS, Hospital de Rangueil), gracias a los fondos de la agencia espacial europea (ESA) y del centro nacional de estudios espaciales (CNES). El estudio consistió en 60 días de hipoactividad utilizando el modelo de encamamiento prologando inclinado (« Head-Down Bed Rest », HDBR). Veinte sujetos sanos fueron reclutados y asignados de manera aleatoria en dos grupos: un

grupo control que recibió una dosis de placebo, y otro grupo que recibió una dosis diaria de cocktail antioxidante/anti-inflamatorio. Este estaba compuesto por 138mg de vitamina E, 530mg de polifenoles, 80µg de selenio, y 2,1g de oméga-3. Biopsias musculares fueron extraídas de los músculos *vastus lateralis* de los sujetos antes del bedrest (*pre*), al final de los 60 días (*post*), y 10 días después de la intervención (*recovery*).

La fuerza muscular de los sujetos fue medida antes y después los 60 días de encamamiento, con la realización de contracciones isométricas maximales realizadas en posición de extensión/flexión de cadera y rodilla. El análisis immuno-histoquímico fue realizado sobre cortes transversales e 10µm de espesor realizadas con criostato. Un marcado con laminina permitió la determinación de las CSA de las fibras, y los anticuerpos específicos a los distintos tipos de miosina (MyHC I, II, IIa) permitieron la evaluación de la tipología muscular. El nivel de proteínas carboniladas así como los western-blot fueron realizados siguiendo los mismos protocolos que los del estudio I. Los controles de carga utilizados para este estudio se hicieron gracias a la tecnología "strain-free" permitida por la utilización de geles pre-fundidos (Bio-Rad).

Resultados: Los resultados principales de este estudio demostraron que después dos meses de encamamiento, todos los sujetos presentaron un DM caracterizado por una pérdida de fuerza muscular (miembros inferiores) y una atrofia de las fibras musculares. La toma del cocktail no previno la de pérdida de masa muscular. Por el contrario, las fibras tipo II de los sujetos suplementados fueron más atrofiadas que las de los sujetos controles. No obstante, la suplementación limitó el aumento importante del número de fibras de tipo IIx, comúnmente observado tras una inmovilización. Los resultados de los análisis bioquímicos permitieron destacar un potencial efecto protector de la suplementación respecto a los daños oxidativos musculares (proteínas carbonlinadas y peroxidacion lipídica). Sin embargo, los resultados de varios actores de contenido mitocondrial, metabolismo oxidativo, y vías de señalización del balance proteico (síntesis, degradación y autofagia), indican una ineficacia del cocktail en la prevención del DM. Es especialmente en el periodo de recarga (entre las biopsias *post* y *recovery*), que los efectos de la suplementación parecen ser los más desfavorables para las adaptaciones moleculares inducidas por la removilización. Este estudio resalta la complejidad de los mecanismos de regulación del equilibrio redox, y formula interrogantes respecto al tipo de intervenciones nutricionales apropiadas. Los efectos negativos de la

suplementación observados en nuestro estudio podrían ser explicados por una interacción entre los distintos compuestos del cocktail, generando perturbaciones a nivel de algunas vías de señalización. La segunda hipótesis es seguramente un desequilibrio del balance redox a favor de las moléculas antioxidantes. En vez de haber restablecido el equilibrio, la fuerte dosis de antioxidantes y la duración del tratamiento han podido provocar una fuerte inhibición de los ROS, cuyo concentración sabemos que, a dosis bajas, es necesaria para el buen funcionamiento de varias vías de señalización. Así, en lugar de reducir el estrés oxidativo inducido por la hipoactividad, el cocktail ha podido inducir un estado de "estrés reductor" (Narasimhan & Rajasekaran, 2015). Esta explicación es acorde con los efectos observados en el campo del ejercicio físico, en el cual la toma de antioxidantes es perjudicial para el rendimiento debido a la inhibición de los mecanismos de adaptación (M.-C. Gomez-Cabrera et al., 2008; M. C. Gomez-Cabrera et al., 2012).

Conclusión: Finalmente, las conclusiones de nuestros dos estudios proporcionaron ideas sobre la estrategia de modulación antioxidante adecuada para la prevención del DM. Probablemente la mejor opción podría ser la de buscar estrategias dirigidas a la estimulación endógena de los sistemas de defensa (resultados del estudio I), en lugar de enfocarse en la toma exógena de sustancias antioxidantes

(resultados estudio II). Sin embargo, la complejidad de las vías de señalización redox requiere una mejor comprensión para optimizar las medidas a tomar contra la pérdida muscular. Es importante destacar que los mecanismos que conllevan al DM pueden ser distintos según las situaciones (hipoactividad del sujeto sano vs envejecimiento), y su comprensión permitirá generar estrategias preventivas óptimas.

RESUME EN FRANCAIS

Introduction : Le système musculaire joue un rôle primordial dans l'homéostasie de l'organisme. Il est impliqué dans différentes fonctions indispensables aux activités de la vie quotidienne telles que la production de mouvement, la locomotion, le maintien postural et l'équilibre. La qualité du tissu musculaire est donc primordiale dans le maintien de la qualité de vie et, à long terme, à la longévité. Cependant, la généralisation d'un mode de vie sédentaire d'une part, et le vieillissement de la population d'autre part, sont deux phénomènes en expansion depuis le siècle dernier dont le point commun est une perte de quantité (masse) et de qualité (fonction) musculaire. Les muscles striés squelettiques représentent à eux seuls près de 40% de notre masse corporelle, et sont caractérisés par une haute plasticité, c'est-à-dire la capacité à s'adapter à différents stress environnementaux (Harridge, 2007). Le terme de déconditionnement musculaire traduit un dérèglement de l'équilibre homéostasique et est la conséquence d'un grand nombre de facteurs. Parmi eux, on trouve la sédentarité, l'avancée en âge, l'immobilisation chronique, l'environnement en impesanteur ou encore certaines pathologies (Degens & Alway, 2006). Le déconditionnement musculaire conduit à une altération de la fonction musculaire, et peut être apprécié par de

nombreuses caractéristiques. La première est la perte de masse musculaire, évaluée au niveau des fibres musculaires par une diminution de leur surface de section transversale (CSA). Celle-ci est associée à un shift myotypologique caractérisé par une diminution des fibres de type I au phénotype oxydatif, au profit des fibres de types II au phénotype glycolytique. Ces modifications structurales sont accompagnées d'une perte de qualités contractiles, en partie responsable de la diminution de force et de puissance musculaire. De plus, d'autres facteurs rentrent en jeu, comme par exemple les facteurs nerveux ou encore l'accumulation de cellules adipeuses intermusculaires (appelées IMAT), (K M Baldwin et al., 2013; Bodine, 2013; Gillis & MacDonald, 2005). La dérégulation des voies signalétiques impliquées dans le maintien de la masse musculaire est largement responsable des altérations observables au niveau fonctionnel. Cet équilibre est basé sur la balance protéique, qui contrôle les voies anaboliques et cataboliques dans le but de maintenir un contenu protéique optimal (Sandri, 2008; Schiaffino, Dyar, Ciciliot, Blaauw, & Sandri, 2013). En situation de déconditionnement musculaire, ces voies de synthèse et de dégradation protéique sont perturbées, conduisant au phénomène d'atrophie musculaire.

Dans les dernières décennies, la recherche scientifique a mis au point de nombreux modèles expérimentaux (cellulaires, animaux et

humains), permettant d'étudier les processus sous-jacents au déconditionnement musculaire. Leur utilisation est essentielle à la compréhension des mécanismes moléculaires impliqués et à l'identification de potentielles cibles, dans le but de tester des mesures de prévention de la perte de masse et de force musculaire.

Le déconditionnement musculaire lié à l'avancée en âge est appelé sarcopénie. Elle est définie par une perte progressive et généralisée de masse et de force musculaire, associée à une faible performance physique et une mobilité réduite (A J Cruz-Jentoft et al., 2010; Fielding et al., 2011; John E. Morley et al., 2011; Muscaritoli et al., 2010). La sarcopénie constitue un facteur d'apparition majeur du syndrome de fragilité (« frailty »), avec qui elle partage plusieurs caractéristiques phénotypiques communes (Cederholm, 2015; Landi et al., 2015). La fragilité est considérée comme un déclin progressif des systèmes physiologiques lié à l'avancée en âge. Elle est le résultat d'une diminution des capacités intrinsèques de résistance aux stress, augmentant les risques d'effets négatifs sur la santé comme l'hospitalisation, la perte d'autonomie ou encore un décès précoce (Cesari et al., 2014; Clegg et al., 2013; Nascimento et al., 2019; Rodriguez-Manas & Fried, 2015). En dépit de la variété d'aspects que couvre le syndrome de fragilité, il est affirmé que la fonction physique et musculaire y tient un rôle primordial (Abellan van Kan et al., 2009;

Daniels et al., 2008; Ferrucci et al., 2004). Fried et al. (2001) ont établi un phénotype de sujet "fragile", qui inclut les critères de sélection suivants: perte de poids non-intentionnelle, épuisement, faiblesse musculaire, faible vitesse de marche et niveau d'activité physique. Les sujets sont considérés comme étant « fragiles » s'ils complètent au minimum 3 de ces 5 critères. Cette évaluation est la plus commune pour évaluer la fragilité dans le domaine clinique. Avec l'augmentation de la prévalence de ce syndrome au cours des dernières décennies, il est primordial de comprendre les mécanismes moléculaires qui lui sont associés (Puts et al., 2017). Pour cela, le développement de modèles expérimentaux sur l'animal nous offre les moyens d'étudier ces mécanismes et de tester des stratégies visant à retarder son apparition.

La balance redox définit un équilibre entre les molécules pro-oxydantes et anti-oxydantes, essentiel au bon fonctionnement de l'organisme. Cet état stable est intimement lié au fonctionnement des différentes voies signalétiques régulant de nombreuses fonctions physiologiques, comme la production d'énergie, la fonction immunitaire ou encore la contraction musculaire (Reid et al., 1993; H. Sies et al., 1985; H Sies, 1997; Helmut Sies, 2000). Les espèces oxygénées réactives (ROS) sont le produit du métabolisme cellulaire des organismes vivants (B Halliwell, 1987; M. Valko et al., 2006). Selon

leur concentration dans l'organisme, ils jouent un double rôle, pouvant être néfaste ou bénéfique. En effet, à doses faibles à modérées, les ROS sont essentiels à de nombreux processus cellulaires, mais engendrent des dommages macromoléculaires quand ils sont présents à hautes concentrations (Barry Halliwell & Gutteridge, n.d.; Marnett, 1999; Siems et al., 1995; Earl R Stadtman, 2004; Marian Valko et al., 2007; Wang et al., 1996). Pour contrer les effets délétères de l'excès de ROS, l'organisme peut compter sur un puissant système de défenses anti-oxydantes. En 1995, Halliwell proposait une définition d'un antioxydant comme étant « une substance qui, quand elle est présente à faible concentration comparée à celle des substrats oxydables, retarde significativement ou prévient l'oxydation de ces substrats ». Les molécules anti-oxydantes ont pour fonction de lutter contre la formation de ROS et de bloquer les attaques de radicaux libres. Elles ont la capacité de séquestrer les métabolites réactifs, diminuant ainsi leur niveau de réactivité, et facilitent également la réparation des dommages induits par les ROS (Birben et al., 2012; Poljsak et al., 2013). Les défenses anti-oxydantes se définissent par l'action d'enzymes (catalase, glutathion peroxydase ou superoxyde dismutase), mais également par l'action d'agents non-enzymatiques parmi lesquels on trouve le glutathion, les vitamines C, E, les caroténoïdes ou encore les polyphénols.

Le concept de stress oxydant a d'abord été décrit par Sies (1985) et traduit un taux excessif de ROS, résultant en un déséquilibre entre leur production et leur élimination (H Sies, 1983). Une perturbation du statut redox engendre des dommages irréversibles aux protéines, lipides, et à l'ADN, qui *in fine* altèrent les fonctions physiologiques et contribuent au développement de nombreuses pathologies (Bokov et al., 2004; Cross et al., 1987).

L'enzyme glucose-6-phospate déshydrogénase (G6PD) a été caractérisée pour la première fois par Warburg & Christian en 1932, qui ont découvert son implication dans les fonctions redox au niveau des globules rouges. La G6PD est une enzyme exprimée dans toutes les cellules de l'organisme, et constitue la première étape de la voie des pentoses phosphates. Elle permet la production de NADPH, dont la concentration est déterminante pour le fonctionnement de plusieurs systèmes antioxydants, en particulier celui du glutathion. En effet, NADPH est un co-facteur de la glutathion réductase (GR), qui catalyze la réduction du glutathion oxydé (GSSG) en glutathion réduit (GSH) (Mannervik, 1987; Meister, 1988; Scott et al., 1993; Helmut Sies, 1999). GSH est un acteur redox central de la plupart des organismes aérobies. Il ne nécessite pas d'intervention enzymatique pour exercer son pouvoir anti-oxydant, et peut directement éliminer certaines espèces réactives. Etant donnée l'implication de la G6PD dans les

défenses anti-oxydantes, un déficit de cette enzyme constitue un facteur de risque important dans le développement de nombreuses pathologies (favisme, diabète, hypertension, arrêt cardiaque…) (Bocchetta, 2003; Hecker et al., 2013; Heymann et al., 2012; Hwang et al., 2018). Le déficit en G6PD est la perturbation enzymatique la plus répandue au niveau mondial, touchant plus de 400 millions de personnes (Nkhoma et al., 2009; Peters & Van Noorden, 2009). Cependant, son rôle dans le maintien et l'homéostasie du tissu musculaire n'a été que très peu étudié.

Le déconditionnement musculaire, et plus particulièrement les mécanismes moléculaires impliqués dans la perte de masse qui le caractérise, sont influencés par les ROS via un contrôle redox du turnover protéique. En effet, la littérature a montré que le stress oxydant contribue à l'atrophie musculaire d'une part en inhibant la voie de synthèse PI3K-Akt-mTORC1, et d'autre part en stimulant la protéolyse via l'activation du système autophagique et du système ubiquitine-protéasome (Powers et al., 2016).

En tant que processus universel, intrinsèque, progressif et délétère, le vieillissement a été largement étudié et plus de 300 théories ont été proposées (Medvedev, 1990). Il y a quelques années, la revue de Viña

et al. (2007) en a proposé une compilation, indiquant la plus connue de toutes : « la théorie des radicaux libres du vieillissement ». Harman (1956) fut le premier à émettre l'hypothèse que les ROS étaient responsables des dommages moléculaires associés à l'avancée en âge. Depuis, la théorie a évolué et des multiples études, à la fois en sa faveur, et contre celle-ci, ont vu le jour (Viña et al., 2013).

De nombreuses données dans la littérature ont mis en évidence une dérégulation de la balance redox pendant le vieillissement. L'augmentation de la production et l'accumulation de dommages oxydatifs ont été reportés dans le tissu musculaire d'organismes âgés (Drew et al., 2003; Sastre et al., 2003; Vasilaki et al., 2006). De plus, une diminution significative des défenses anti-oxydantes chez la personne âgée pourrait contribuer à l'installation de la sarcopénie, et plus particulièrement au syndrome de fragilité (Baumann et al., 2016; M. J. Jackson, 2015; Kerksick & Zuhl, 2015; Scicchitano et al., 2018). Récemment, des chercheurs de notre équipe ont souligné que le stress oxydant était en réalité associé à la fragilité, plutôt qu'au vieillissement en soi, (Inglés et al., 2014). Ainsi, « la théorie des radicaux libres de la fragilité » est apparue, fournissant un rationnel scientifique aux interventions tournées vers la protection des dommages oxydatifs pour retarder la fragilité (Viña, 2019; Viña et al., 2018). En effet, plusieurs études cliniques ont démontré une

corrélation entre le statut fragile des sujets, et une élévation de certains marqueurs de dommages oxydatifs (isoprostanes, 4-HNE, MDA, 8-OHdG), (Cesari et al., 2012; C. K. Liu et al., 2016; Serviddio et al., 2009; Wu et al., 2009).

Les interventions visant à augmenter les défenses anti-oxydantes comme stratégies contre le déconditionnement musculaire (en situation de vieillissement ou d'inactivité) constituent un domaine actif de recherche (Bonetto et al., 2009; Janna R. Jackson et al., 2010; Ryan et al., 2011). Chez des sujets âgés, plusieurs études démontrent une corrélation positive entre le niveau plasmatique d'anti-oxydants (vitamines C, E, caroténoïdes) et des paramètres de fonction musculaire (force, vitesse de marche) (Ble et al., 2006; A. Das et al., 2019; J.-S. Kim et al., 2010; Saito et al., 2012; Semba et al., 2003). En revanche, la majorité des études utilisant une supplémentation nutritionnelle avec des telles molécules, a échoué à démontrer un impact positif sur les pertes de force, de masse, ou de performance musculaire liées à l'âge (Bobeuf et al., 2011; Marzani et al., 2008; Nalbant et al., 2009). D'autre part, dans la lutte contre le déconditionnement musculaire induit par une réduction d'activité, des supplémentations en diverses substances anti-oxydantes (vitamine E, polyphénols, curcuma, β-carotène) ont montré des effets mitigés. Néanmoins, une base solide d'études met en avant leurs

effets bénéfiques contre la perte de masse et de force musculaire (Beytut et al., 2018; Demiryürek S, 2004; Duarte & Soares, 1997; Kondo et al., 1991; Kondo, Miura, Nakagaki, et al., 1992; Momken et al., 2011; Ogawa et al., 2013; Servais et al., 2007).

Objectifs : L'objectif principal de cette thèse était d'évaluer l'impact d'une modulation des défenses anti-oxydantes, sur la prévention du déconditionnement musculaire. Celui-ci a été étudié sous deux angles, le premier lié à l'avancée en âge et le second à l'hypoactivité. En effet, cette thèse a été conduite dans deux laboratoires et a ainsi permis l'élaboration de deux projets expérimentaux. Le premier a été conduit sur un modèle animal âgé, et s'est centré sur la prévention de la perte de fonction musculaire liée à la fragilité, tandis que le second projet s'est intéressé chez l'homme au déconditionnement musculaire induit par une hypoactivité prolongée. Concernant la modulation des défenses anti-oxydantes, chaque étude a pu employer deux stratégies distinctes. La première étude a utilisé une manipulation génétique pour stimuler un acteur clé du système anti-oxydant endogène, alors que la seconde étude a étudié l'impact d'une supplémentation nutritionnelle en substances anti-oxydantes.

Etude I : L'étude I de cette thèse est intitulée: "Sur-expression de G6PD : effets sur la fragilité chez des souris âgées ». Le but de cette étude était d'évaluer la fragilité dans une cohorte de souris âgées, utilisant un groupe de souris WT et un groupe de souris transgéniques (G6PD-Tg) sur-exprimant l'enzyme G6PD.

Matériel et méthodes : Ces souris transgéniques ont été générées au Centre Espagnol National de recherché contre le cancer (CNIO), grâce à l'insertion d'un fragment génomique humain contenant le gène G6PD. De l'âge de 18 à 26 mois, nous avons réalisé une évaluation longitudinale de 5 paramètres fonctionnels (perte de poids, force maximale, coordination motrice, vitesse maximale et temps de course atteint lors d'une épreuve de course sur tapis), et avons ensuite calculé un score établissant la fragilité des animaux dans chacun des groupes. Grâce aux échantillons musculaires de souris âgées de 21 mois, nous avons évalué divers paramètres moléculaires liés au stress oxydant, à la qualité du tissu musculaire ainsi qu'à l'expression de gènes spécifiques.

Les niveaux de glutathion réduit (GSH) et oxydé (GSSG) ont été mesurés par une méthode spéctrophotométrique utilisant le N-ethylmaleimide. La carbonylation de protéines a été évaluée en suivant les instructions de l'« oxyblot protein oxidation detection kit ».

La peroxydation lipidique a été déterminée par HPLC (chromatographie en phase liquide à haute performance), grâce à la détermination des niveaux de malondialdehyde (MDA). La technique du western-blot a été utilisée pour mesurer le contenu de diverses protéines. Une analyse histologique a été effectuée sur les coupes transversales de muscles de souris très âgées pour évaluer la CSA des fibres. L'analyse transcriptomique a débuté par une extraction d'ARN totaux des échantillons musculaire avec la méthode du Trizol. Puis, la synthèse d'ADN et d'ARN complémentaires des échantillons a été réalisée en suivant les instructions de Kit « WT Plus Reagent Kit Manual » (Thermo Fisher), générant une puce à ADN (« microarray ») contenant plus de 20000 gènes. Les analyses statistiques ont été réalisées avec les logiciels Statistica ou GraphPad prism, avec une significativité placée à $p<0.05$. La normalité des distributions était vérifiée par le test de Shapiro-wilk. Pour comparer deux groupes, le test-t de student était utilisé, ou le test de Mann-Whitney dans le cas d'une distribution non normale. Pour le « frailty score », les différences ont été testées par le test Chi^2 de Pearson.

Résultats : Les résultats de cette étude montrent que les souris âgées G6PD-Tg sont moins fragiles que leurs congénères WT. Nous avons pu observer un taux plus élevé de GSH dans les muscles d'animaux sur-exprimant G6PD, ainsi qu'un ratio GSSG/GSH plus faible,

indiquant une amélioration du système antioxydant du glutathion. De plus, les analyses biochimiques ont mis en évidence chez les souris G6PD-Tg une élévation de différents marqueurs indiquant une meilleure qualité musculaire (meilleur contenu mitochondrial, moindres infiltrations graisseuses intermusculaires, apoptose réduite). Enfin, l'analyse transcriptomique a permis de détecter une surexpression de gènes impliqués dans les mécanismes de la chaîne respiratoire, la phosphorylation oxydative ainsi que du métabolisme du glutathion. Cette étude a permis de définir le premier modèle expérimental qui retarde l'apparition de la fragilité. Ainsi, des interventions nutritionnelles ou encore l'exercice physique visant l'activation de l'enzyme G6PD sont des pistes potentielles à investiguer dans la recherche de stratégies de prévention de perte de fonction musculaire chez la personne âgée.

Etude II : L'étude II de cette thèse est intitulée: « effets d'une supplémentation en anti-oxydants sur le déconditionnement musculaire induit par l'hypoactivité ». Cette étude avait donc pour but d'évaluer les effets d'une prise de cocktail anti-oxydant sur le déconditionnement musculaire induit par 2 mois d'alitement prolongé.

Matériel et méthodes : L'expérimentation a été conduite au sein de la clinique spatiale de Toulouse (Medes-IMPS, Hôpital de Rangueil), sous l'égide de l'agence spatiale européenne (ESA) et financée pour notre partie expérimentale par le Centre National d'Etudes Spatiales (CNES). L'étude consistait en 60 jours d'hypoactivité utilisant le modèle d'alitement prolongé avec déclivité(« Head-Down Bed Rest », HDBR). Vingt sujets sains ont été recrutés et placés de manière aléatoire dans deux groupes : un groupe contrôle recevant une dose placebo, et un groupe recevant une dose quotidienne d'un cocktail anti-oxydant/anti-inflammatoire. Celui-ci était composé de 138mg de vitamine E, 530mg de polyphénols, 80µg de sélénium, et 2,1g d'oméga-3. Des biopsies musculaires ont été prélevées dans les muscles *vastus lateralis* des sujets avant le bedrest (*pre*), à la fin des 60 jours (*post*), et 10 jours après la remobilisation (*recovery*).

La force musculaire des sujets a été mesurée avant et après les 60 jours d'alitement, grâce à la réalisation de contractions maximales isométriques réalisée en position d'extension/flexion de hanche et de genoux. L'analyse immuno-histochimique a été réalisée sur des coupes transversales de 10µm d'épaisseur réalisées au cryostat. Un marquage laminine a permis la détermination des CSA des fibres, tandis que les anticorps spécifiques aux différents types de myosines (MyHC I, II, IIa) ont permis d'évaluer la typologie musculaire. Le

niveau de protéines carbonylées ainsi que les western-blot ont été réalisés en suivant les mêmes protocoles que pour l'étude I. Les contrôles de charge utilisés pour cette étude se sont fait grâce à la technologie « stain-free » permise par l'utilisation de gels pré-coulés (Bio-Rad).

Résultats : Les résultats principaux de cette étude montrent qu'après deux mois d'alitement, tous les sujets présentaient un déconditionnement musculaire caractérisé par une perte de force musculaire (membres inférieurs) et une atrophie des fibres musculaires. La prise du cocktail n'a pas permis la prévention de perte de masse musculaire. Au contraire, les fibres de type II des sujets supplémentés étaient davantage atrophiées que celles des sujets contrôles. En revanche, la supplémentation a permis de limiter l'augmentation importante du nombre de fibres de type IIx, couramment observée suite à une immobilisation. Les résultats des analyses biochimiques ont permis de soulever un potentiel effet protecteur de la supplémentation concernant les dommages oxydatifs musculaires (protéines carbonylées et peroxidation lipidique). En revanche, les résultats obtenus sur de nombreux acteurs de contenu mitochondrial, métabolisme oxydatif, et voies signalétiques de la balance protéique (synthèse, dégradation et autophagie) indiquent l'inefficacité du cocktail sur la prévention du déconditionnement

musculaire. C'est plus particulièrement sur la période de remise en charge (entre les biopsies *post* et *recovery*), que les effets de la supplémentation semblent être les plus néfastes aux adaptations moléculaires induites par la remobilisation. Cette étude souligne la complexité des mécanismes de régulation de la balance redox, et soulève des interrogations quant à la mise en place d'interventions nutritionnelles appropriées. Les effets négatifs de la supplémentation observés dans notre étude pourraient être expliqués par une interaction entre les différents composés du cocktail, engendrant des perturbations au niveau de voies signalétiques moléculaires. La seconde hypothèse est certainement un dérèglement de la balance redox en faveur des molécules anti-oxydantes. Au lieu d'avoir rétabli un équilibre, la forte dose d'anti-oxydant et la durée du traitement ont pu favoriser une trop forte inhibition des ROS, dont on sait que leur concentration, à faible dose, est nécessaire au bon fonctionnement de plusieurs voies signalétiques. Ainsi, au lieu de réduire le stress oxydant induit par la période d'hypoactivité, le cocktail a pu induire un état de « stress réducteur » (Narasimhan & Rajasekaran, 2015). Cette explication rejoint les effets observés dans le cadre de l'exercice physique, où la prise d'anti-oxydants est alors néfaste à la performance, via une inhibition des mécanismes d'adaptations

moléculaires (M.-C. Gomez-Cabrera et al., 2008; M. C. Gomez-Cabrera et al., 2012).

Conclusion : Enfin, les conclusions de ces deux études ouvrent des pistes sur les modulations anti-oxydantes les plus appropriées à utiliser dans le cadre de la prévention du déconditionnement musculaire. Il semble préférable de s'intéresser aux stratégies visant à la stimulation endogène des systèmes de défenses (résultats étude I), plutôt que de se tourner vers une prise exogène de substances anti-oxydantes (résultats étude II). Cependant, la complexité des voies de signalisation redox requiert une meilleure compréhension pour optimiser les contre-mesures visant la prévention du déconditionnement musculaire. Il est important de souligner que les mécanismes conduisant au déconditionnement musculaire peuvent différer selon les situations (hypoactivité du sujet sain *vs* vieillissement), et leur compréhension permet la mise au point de stratégies préventives optimales.

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ANNEX

Table 1: Body weights (grams) of WT and G6PD-Tg mice. All data and Mean ± SEM.

18-20 months		21-22 months		23-34 months		25-26 months	
WT (n=24)	**Tg (n=39)**	**WT (n=17)**	**Tg (n=36)**	**WT (n=13)**	**Tg (n=29)**	**WT (n=11)**	**Tg (n=24)**
43,4	36,6	38,3	39,2	36,8	33,9	38,0	35,1
40,4	36,6	34,7	32,3	34,0	33,6	35,4	32,6
39,9	33,1	34,7	31,4	32,3	32,0	30,7	30,3
39,6	32,3	34,1	29,4	32,3	31,4	29,8	29,5
34,2	31,7	33,4	29,2	31,0	29,0	28,6	28,9
34,0	30,2	33,3	29,1	30,4	28,8	28,5	28,7
33,8	28,7	31,4	28,8	29,0	27,7	28,5	28,6
33,0	28,5	30,9	28,7	28,6	27,4	27,6	28,5
32,2	28,5	30,7	28,7	26,4	27,3	26,4	28,4
32,0	28,2	30,6	28,6	25,5	27,3	25,5	28,2
31,5	27,7	30,1	28,0	25,2	27,2	25,1	28,0
31,3	27,5	30,0	27,6	24,9	26,9		27,7
29,7	27,5	28,5	27,5	24,9	26,9		27,6
29,2	27,3	28,2	27,4		26,8		27,5
29,0	27,1	25,9	27,4		26,7		27,3
28,6	26,9	24,6	27,1		26,5		27,0
28,4	26,7	24,2	27,0		25,8		26,1
27,6	26,6		26,8		25,7		25,7
27,3	26,3		26,7		25,3		25,6
27,1	26,2		26,4		25,2		25,6
26,9	25,9		25,8		24,8		25,0
25,9	25,8		25,7		24,7		24,1
25,2	25,7		25,6		24,6		23,7
24,4	25,4		25,5		24,4		22,3
	25,2		25,4		24,3		
	24,8		25,3		24,1		

	24,7		25,1		23,5		
	24,6		24,8		22,5		
	24,5		24,7		22,2		
	24,5		24,5				
	24,3		24,3				
	24,1		24,1				
	24,0		23,8				
	23,9		23,5				
	23,0		23,4				
	22,9		22,7				
	22,5						
	22,3						
	22,2						
31,4 ± 1	**26,8 ± 0,6**	**30,8 ± 0,9**	**27 ± 0,5**	**29,3 ± 1,1**	**26,8 ± 0,5**	**29,5 ± 1,2**	**27,6 ± 0,6**

Table 2: Maximal grip strength of WT and G6PD-Tg mice. All data and Mean ± SEM.

18-20 months		**21-22 months**		**23-34 months**		**25-26 months**	
WT (n=24)	**Tg (n=40)**	**WT (n=17)**	**Tg (n=36)**	**WT (n=14)**	**Tg (n=29)**	**WT (n=11)**	**Tg (n=24)**
6,6	7,5	5,5	6,8	5,5	6,5	5,4	5,8
5,9	7,1	5,4	6,4	5,3	6,0	5,2	5,7
5,8	6,6	5,2	6,4	4,3	5,6	4,6	5,5
5,8	6,5	5,1	6,3	4,2	5,6	4,0	5,4
5,5	6,3	5,1	6,2	4,1	5,6	3,8	5,1
5,3	6,3	4,9	6,0	4,1	5,6	3,8	4,9
5,0	6,3	4,9	5,9	3,9	5,5	3,5	4,8
4,8	6,2	4,8	5,8	3,7	5,4	3,5	4,7
4,8	6,1	4,7	5,8	3,6	5,4	3,3	4,6
4,8	6,1	4,4	5,6	3,4	5,3	3,1	4,5
4,7	6,0	4,2	5,5	3,3	5,3	2,9	4,5
4,6	5,9	4,1	5,5	3,2	5,2		4,4
4,6	5,9	3,9	5,4	3,0	5,1		4,4

4,1	5,8	3,9	5,4	3,0	5,1		4,1
4,1	5,8	3,7	5,3		5,1		3,8
4,1	5,7	3,6	5,3		5,0		3,7
4,1	5,7	3,2	5,2		5,0		3,6
4,0	5,7		5,1		4,8		3,6
3,9	5,7		5,1		4,8		3,6
3,7	5,6		5,1		4,8		3,4
3,7	5,5		5,0		4,7		3,4
3,6	5,5		4,9		4,7		3,4
3,5	5,4		4,9		4,7		2,8
2,8	5,4		4,9		4,5		2,7
	5,3		4,9		4,4		
	5,2		4,8		4,2		
	5,2		4,8		3,7		
	5,2		4,7		3,6		
	5,2		4,6		3,5		
	5,1		4,5				
	4,8		4,2				
	4,4		4,0				
	4,3		4,0				
	4,3		3,8				
	4,0		3,7				
	3,9		3,6				
	3,7						
	3,5						
	3,4						
	3,3						
4,6 ± 0,2	**5,4 ± 0,2**	**4,5 ± 0,2**	**5,1 ± 0,1**	**3,9 ± 0,2**	**5 ± 0,1**	**3,9 ± 0,3**	**4,3 ± 0,2**

Table 3: Running time (hr:min:sec) achieved during the treadmill test by WT and G6PD-Tg mice. All data and Mean ± SEM.

18-20 months		**21-22 months**		**23-34 months**		**25-26 months**	
WT (n=24)	**Tg (n=39)**	**WT (n=17)**	**Tg (n=36)**	**WT (n=12)**	**Tg (n=28)**	**WT (n=5)**	**Tg (n=16)**

00:27:47	00:26:17	00:24:02	00:24:56	00:23:09	00:21:20	00:17:07	00:20:47
00:25:31	00:26:09	00:21:33	00:24:11	00:22:19	00:20:33	00:17:03	00:20:35
00:24:00	00:25:26	00:19:00	00:23:50	00:22:16	00:19:56	00:16:28	00:20:16
00:23:36	00:23:55	00:18:40	00:23:18	00:20:40	00:19:30	00:12:13	00:19:37
00:23:19	00:23:51	00:18:40	00:23:17	00:19:15	00:19:25	00:11:22	00:19:13
00:23:03	00:23:04	00:18:20	00:22:53	00:18:58	00:18:50		00:18:12
00:22:27	00:22:50	00:18:14	00:22:03	00:18:01	00:18:40		00:16:58
00:21:47	00:22:50	00:17:30	00:21:15	00:14:40	00:18:33		00:16:40
00:20:45	00:22:46	00:17:11	00:20:20	00:14:37	00:18:31		00:14:15
00:20:42	00:22:42	00:16:37	00:20:10	00:14:31	00:18:30		00:14:14
00:20:09	00:22:40	00:16:25	00:19:55	00:14:22	00:18:25		00:14:06
00:19:42	00:22:38	00:15:25	00:19:40	00:12:38	00:18:12		00:13:45
00:19:20	00:22:27	00:14:39	00:18:55		00:18:05		00:13:10
00:18:40	00:22:08	00:14:23	00:18:40		00:18:04		00:13:07
00:18:30	00:22:06	00:12:19	00:18:40		00:17:49		00:11:44
00:17:33	00:21:30	00:10:54	00:18:24		00:17:43		00:11:01
00:17:32	00:21:08	00:10:35	00:18:20		00:17:27		
00:16:50	00:20:48		00:18:20		00:17:02		
00:16:35	00:20:05		00:18:18		00:16:48		
00:16:06	00:20:02		00:18:10		00:16:15		
00:14:06	00:19:33		00:18:10		00:16:03		
00:13:10	00:19:10		00:17:40		00:15:00		
00:12:17	00:19:02		00:17:32		00:14:53		
00:11:27	00:18:50		00:17:30		00:14:25		
	00:18:48		00:16:54		00:14:16		
	00:18:40		00:16:34		00:14:07		
	00:18:29		00:16:34		00:14:04		
	00:18:20		00:16:29		00:12:58		
	00:18:20		00:16:20				
	00:18:15		00:16:15				
	00:18:10		00:16:11				
	00:17:52		00:15:03				
	00:17:50		00:14:15				
	00:17:20		00:13:13				
	00:17:02		00:12:32				

	00:16:32		00:12:17				
	00:16:20						
	00:15:15						
	00:14:10						
00:19:22 ± 00:00:52	**00:20:21 ± 00:00:28**	**00:16:44 ± 00:00:51**	**00:18:32 ± 00:00:32**	**00:17:57 ± 00:01:04**	**00:17:20 ± 00:00:25**	**00:14:51 ± 00:01:15**	**00:16:05 ± 00:00:50**

Table 4: Maximal running speed (m/min) achieved during the treadmill test by WT and G6PD-Tg mice. All data and Mean ± SEM.

18-20 months		**21-22 months**		**23-34 months**		**25-26 months**	
WT (n=24)	**Tg (n=39)**	**WT (n=17)**	**Tg (n=36)**	**WT (n=12)**	**Tg (n=28)**	**WT (n=5)**	**Tg (n=16)**
34,8	34,8	27,6	32,4	30,0	27,6	22,8	27,6
32,4	34,8	32,4	32,4	30,0	27,6	22,8	27,6
32,4	32,4	25,2	30,0	30,0	25,2	22,8	27,6
30,0	30,0	25,2	30,0	27,6	25,2	18,0	25,2
30,0	30,0	25,2	30,0	25,2	25,2	15,6	25,2
30,0	30,0	25,2	30,0	25,2	25,2		25,2
30,0	30,0	22,8	30,0	25,2	25,2		22,8
27,6	30,0	22,8	27,6	20,4	25,2		22,8
27,6	30,0	22,8	27,6	20,4	25,2		20,4
27,6	30,0	22,8	27,6	20,4	25,2		20,4
27,6	30,0	20,4	25,2	20,4	25,2		20,4
25,2	30,0	20,4	25,2	18,0	25,2		18,0
25,2	30,0	20,4	25,2		25,2		18,0
25,2	30,0	18,0	25,2		25,2		18,0
25,2	30,0	15,6	25,2		22,8		15,6
22,8	27,6	15,6	25,2		22,8		15,6
22,8	27,6	15,4	25,2		22,8		
22,8	27,6		25,2		22,8		
22,8	27,6		25,2		22,8		
22,8	27,6		25,2		22,8		
20,4	25,2		25,2		22,8		
18,0	25,2		22,8		20,4		
18,0	25,2		22,8		20,4		

15,6	25,2		22,8		20,4		
	25,2		22,8		20,4		
	25,2		22,8		20,4		
	25,2		22,8		20,4		
	25,2		22,8		18,0		
	25,2		22,8				
	25,2		22,8				
	25,2		22,8				
	22,8		20,4				
	22,8		20,4				
	22,8		18,0				
	22,8		18,0				
	22,8		18,0				
	22,8						
	20,4						
	20,4						
25,7 ± 1,1	**27,1 ± 0,6**	**22,8 ± 1,1**	**24,9 ± 0,6**	**24,4 ± 1,3**	**23,5 ± 0,5**	**20,4 ± 1,5**	**21,9 ± 1,0**